Studies on the biosynthesis of 1-deoxynojirimycin.

by

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DECLARATION.

The work described in this thesis is the original of the author except where acknowledgement has been made to the results and ideas of others.

The work has been carried out at the departments of Chemistry and Biological Sciences between October 1st 1988 and September 30th 1991. It has not previously been submitted for a degree at any other institution.

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NUMEROUS ORIGINALS
IN COLOUR

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ABSTRACT.

1-Deoxynojirimycin (DNJ) is a 1-deoxy-glucose analogue and glucosidase inhibitor which has also been found to inhibit replication of Human Immunodeficiency Virus.

It is known to be produced by several strains of *Streptomyces lavendulae* and in the present study, *Streptomyces subrutilus* 445 has also been identified as a producer of DNJ and the 1-deoxy-mannose analogue, 1-deoxymannojirimycin (DMJ). DNJ can be assayed in fermentation samples by virtue of its inhibition of pig kidney trehalase, after the removal of the interfering inhibitor nojirimycin by a heat and acid treatment. DNJ and nojirimycin inhibit this trehalase with Ki values of 3.43×10^{-6} and 2.6×10^{-5} M respectively.

The production of DNJ in defined media is subject to supression by glucose and phosphate. Addition of ammonium sulphate to the cultures also inhibits DNJ production although it is unclear whether this is purely a supressive effect. Glucose and starch are the best carbon sources and proline the best nitrogen source in defined media containing only one major carbon and one major nitrogen source.

Glucose is the biosynthetic precursor to both DNJ and DMJ and a pathway is proposed which involves a glucose to fructose isomerisation, an inversion of the sugar backbone, and an epimerisation of nojirimycin B to nojirimycin.

ABBREVIATIONS.

Glc	Glucose.
Man	Mannose.
NAG	N-Acetyl glucosamine.
AZT	3'-Azido-3'deoxythymidine.
M	Molar.
mol	Moles.
Ki	Inhibition constant.
Km	Michaelis constant.
HPLC	High Performance Liquid Chromatography.
GC	Gas Chromatography.
U	Units.
l	Litres.
m	Metres.
min.	Minutes.
rpm.	Revolutions per minute.
h	Hours.
g	Grams.
t.l.c.	Thin Layer Chromatography.
rf.	Relative flow-rate.
ft.	Feet.
nmr	Nuclear Magnetic Resonance.
ATCC	American Type Culture Collection.
°C	Degrees celsius.
ppm	Parts per million.
ppGpp	Guanosine 3'diphosphate 5'diphosphate.
pppGpp	Guanosine 3'-diphosphate 5'triphosphate.
cfu	Colony forming units.

ATP	Adenosine 5'-triphosphate.
t	Time.
Tris	Tris(hydroxymethyl)aminomethane.
EDTA	Ethylenediaminetetraacetic acid.
RASS	Reduced arginine, starch and salts medium.
EMS	Ethyl methane sulphonate.

"Under the most rigorously controlled conditions of temperature, pH, aeration, and nutrient concentration, the organism will do as it damn well pleases."

S.J. Hochauser, 1983.

CHAPTER 1. INTRODUCTION.

1.1. *STREPTOMYCES.*

Streptomyces, a genus of the Actinomycetes, are Gram positive filamentous bacteria which are found ubiquitously in soil (Kutzner, 1981) and have also been isolated from fresh water (Burman, 1973) and marine (Okazaki and Okami, 1976) environments. *Streptomyces* are highly adapted to their natural environment, being able to utilise a wide range of carbon sources and withstand long periods of adverse conditions such as frost, drought and flooding. Their life cycle, shown in Figure 1.1., involves much differentiation, resulting in the development of a mycelium of hyphae and the subsequent production of spores which are desiccation but not heat resistant. This enables them to cope with the range of conditions that they are likely to meet in their natural environment.

A few *Streptomyces* species, such as *S.scabies* which cause common scab disease of potatoes are pathogenic (Kutzner, 1981), but the industrial importance of this group of organisms lies in their ability to produce a large number of structurally diverse secondary metabolites.

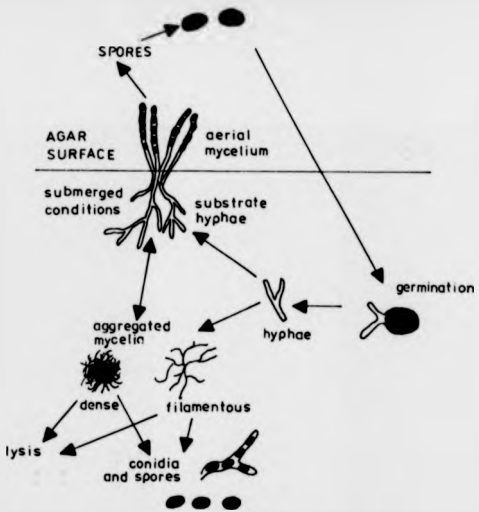


Figure 1.1. Life cycle of *Streptomyces*. (Taken from Grafe, 1989).

1.2. SECONDARY METABOLITES AND THEIR ROLE IN THE PRODUCING ORGANISM.

The metabolic pathways of organisms such as the Streptomycetes can be divided up into so-called primary and secondary metabolism. Whereas primary metabolism consists of reactions which are essential for the cell, such as those involved in respiration, protein synthesis and DNA

replication, the reactions and products of secondary metabolism are not vital for the survival and replication of the cell. In nature secondary metabolism is confined to only a few taxonomic groups, most notably the higher plants, the fungi and the Actinomycetes. Of the Actinomycetes, the *Streptomyces* genus is especially productive. A recent estimate states that out of 6152 known secondary metabolites from Actinomycetes, 4876 are produced by members of the *Streptomyces* genus (Goodfellow and O'Donnell, 1989). Many secondary metabolites have been found to be active as antibacterial or antifungal agents and the exploitation of these and other non-antibiotic secondary metabolites by man will be discussed later.

The products of secondary metabolism are structurally diverse and aspects of the genetics and biochemistry of their production have been studied. Their function and evolution in the producing organism is generally poorly understood although the large amount of genetic material and energy required to make them bears testament to their value to their producers.

Zahner has proposed (Zahner *et al.*, 1982b, Zahner *et al.*, 1983), that secondary metabolism acts as a stage for the random generation of new metabolites in an organism. If the new metabolite is advantitious to the organism, the ability to make it will be maintained in the organism and if it is harmful, then it will be lost. If the product is neither of these, then the ability to make the metabolite may be

maintained long enough for another step to be added onto the pathway thereby making another product which might find a use in the producing organism.

The genetic instability seen in *Streptomyces genes* (Cullum *et al.*, 1989; Mathumathi *et al.*, 1990; Demuyter *et al.*, 1991) involves the deletion and amplification of pieces of the genome and might provide a mechanism for the evolution of such pathways. Indeed, homology between the genes for two distinct amidino transferases involved in the biosynthesis of streptomycin, and isopenicillin N synthetase and the expandase catalysing consecutive steps in the biosynthesis of β-lactams in *Cephalosporium acremonium* and *Streptomyces clavuligerus* (Hunter and Baumberg, 1989) might be seen as evidence for the involvement of amplification followed by divergent evolution of these genes to produce the pathways as they exist today. Evolution of the same genes in different organisms to produce enzymes with slightly different substrate specificities or activities might explain the occurrence of different, yet structurally related, metabolites in different species. During the evolution of pathways to secondary metabolites it would be important to produce a gene with a resistance capability, although this might originally have been only a secondary function prior to the inclusion of a step producing an antibiotic product. The existence of resistance genes which also play a biosynthetic function and ones acting by the modification of the antibiotic (Cundliffe, 1989) tallies well with this

evolutionary path but another route must be hypothesised for those using other mechanisms, such as the modification of a target site.

It has been suggested that antibiotic production pathways are very old and that they have originated from ones in primary metabolism (Piepersberg, 1991). Evidence for this origin is clear in the case of the biosynthesis of polyketides. The process is catalysed by an enzyme system which has a lot of similarities, both in the reactions catalysed and in the enzyme components catalysing them to the Type II fatty acid synthetases seen in the prokaryotes (Hunter and Baumberg, 1989).

The horizontal transfer of genes involved in secondary metabolism, was also hypothesised by Zahner (Zahner *et al.*, 1982). There is evidence for the transfer of genetic material between organisms which live in close contact in the soil, such as actinomycetes, fungi and higher plants. For example, β-lactam antibiotics are found in both *Streptomyces* and fungi such as *Cephalosporium* and the biosynthetic pathway is the same in both groups. The genes for β-lactam biosynthesis in both Streptomyces and fungi are clustered (Smith *et al.*, 1990) and very close homology between isopenicillin N synthetase genes from the different groups of producers also suggests a common ancestry (Weigel *et al.*, 1988). In the case of higher plants, deoxynojirimycin and deoxymannojirimycin, which are both made by *Streptomyces lavendulae* (Ezure *et al.*, 1985; Ezure *et al.*, 1988), are found in the roots of the

mulberry (Yagi *et al.*, 1976) and in the tropical bean *Lonchocarpus sp.* (Fellows *et al.*, 1979) respectively. It appears more likely that the wide distribution of the same metabolite between species or genera has occurred through the evolution of the ability to produce that metabolite in one organism and then its transfer to and propagation in other organisms rather than the *de novo* generation of that capability in all those different organisms.

The maintainence of the highly organised and often intricate pathways of secondary metabolism implies that the metabolites and\or the pathways themselves have some function in the producing organism. Many people have tried to determine what this may be. Initially, ideas were based around a single function encompassing all secondary metabolites, eg. as storage products. In 1961, Bu'lock (Bu'lock, 1961) suggested that secondary metabolic pathways, rather than their products, were of importance to the cell, providing a mechanism by which the primary metabolism can be kept "ticking over" whilst the normal growth of the cell is arrested, perhaps due to nutrient limitation. By functioning so as to provide a slow supply of energy and intermediates to the secondary metabolism, the primary metabolic pathways will be maintained in better order to resume normal function when conditions for vegetative growth are restored (Woodruff, 1966).

However, it seems plausible now that a whole series of functions are carried out, with different functions being

fulfilled by different metabolites. These functions may include acting as storage products, detoxification products, shunt metabolites, and providing defense against predators and advantage over competitors at times when they are especially vulnerable such as sporulation. Some may also act to control differentiation events, sequester minerals from the soil and as pathogenic and symbiotic agents (Demain, 1989b; Vining, 1990).

1.3. SECONDARY METABOLITES AND MAN.

Although the role(s) of secondary metabolites in the producing organism remain unclear, their bioactivity is being successfully expoited by man. Plant extracts have been used in healing for many centuries and some metabolites from plants, such as codeine and quinine, are still used in medicine today. This section will concentrate on those secondary metabolites produced by microorganisms for which man has found a use.

Programs screening fermentations, usually of fungi or actinomycetes, against an identified target such as a particular pathogen or an enzyme involved in a key step in the development of a disease may highlight an active compound. These active compounds may themselves not be clinically useful agents, but their isolation and the

elucidation of their structure can provide a useful starting point for the chemical synthesis of a series of new compounds, structurally based on the natural one. The synthetic or semisynthetic analogues may possess greater activity and less toxicity than the original and therefore be more useful. Concern over the environmental effects of chemical pesticides and herbicides used in agriculture has led to an increase in the importance of finding microbial products, which are less environmentally damaging and can replace them.

Secondary metabolites produced by microorganisms can be divided into groups according to their structures. Table 1.1 shows the main groups along with examples of commercially and medically important members of each group.

METABOLITE	APPLICATION
AMINOGLYCOSIDES	
Streptomycin *(Streptomyces griseus)*	Treatment of tuberculosis and urinary tract infections.
Spectinomycin *(S.spectabilis)*	Treatment of gonorrhea.
Kanamycin *(S.kanamyceticus)*	Infections by gram-negative bacteria.
Gentamycins *(Micromonospora spp.)*	Serious infections by gram negative pathogens.
Hygromycin B *(S.hygroscopicus)*	Antihelminth for swine.
NUCLEOSIDES	
Nikkomycins *(S.tendae)*	Antifungal insecticides.
Herbicidins	Herbicides.
Polyoxins *(S.cacaoi)*	Protection of fruit and vegetable crops against fungal diseases.
MACROLIDES	
Erythromycin *(Saccharopolyspora erythraea)*	Used against gram positive infections especially by *Legionella* and *Chlamidia*.
Tylosin *(Streptomyces fradiae)*	Growth promoter for pigs and poultry. Treatment of respiratory infections in animals.
Avermectins *(S.avermitilis)*	Antihelminths and insecticides.
PEPTIDES	
Phosphinothricin *(S.viridochromogenes)*	Herbicide, inhibitor of glutamine synthetase.
Actinomycin *(S.antibioticus)*	Antitumor agent.
Staphylomycin *(S.virginiae)*	Feed additive for swine and poultry.
Cyclosporin A *(Tolypocladium inflatum)*	Immunosuppressor used to prevent rejection of transplanted organs.
Bleomycin *(S.verticillus)*	Cancer chemotherapy.

Table 1.1. Some applications of secondary metabolites.

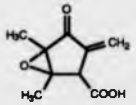
TETRACYCLINES	
Chlortetracycline *(S.aureofaciens)*	Growth stimulant in animals and treatment of infections by gram positive cocci.
Oxytetracycline *(Streptomyces spp.)*	Treatment of acne.
POLYENES	
Nystatin *(S.noursei)*	*Candida* infections of mucous membranes
Candicidin *(S.griseus)*	Candidial vaginitis.
Amphotericin B *(S.nodosus)*	Severe aspergillicosis, hystoplasmosis and coccidioidomycosis.
ANSAMYCINS	
Herbimycins	Fungicidal herbicides.
Rifampicin (Derivative of rifamycin from *Nocardia*)	Treatment of tuberculosis and leprosy.
ANTHRACYCLINES	
Adriamycin *(S.peucetius)*	Chemotherapy for breast cancer and acute leukemia.
Daunomycin *(S.peucetius)*	Cancer chemotherapy.
POLYETHERS	
Monensin *(S.cinnamonensis)*	Coccidiostat, insecticide and growth promoter in ruminants.
Salinomycin *(S.albus)*	Coccidiostat, insecticide and growth promoter in ruminants.
β-LACTAMS	
Clavulanic acid *(S.clavuligerus)*	β-Lactamase inhibitor used to enhance the effect of amoxycillin in the drug "Augmentin".
OTHERS	
Cycloserine *(Streptomyces spp.)*	Treatment of tuberculosis.
Chloramphenicol *(S.venezuelae)*	Used against *Salmonella*, typhoid, bacterial meningitis and for opthalmological infections.

Table 1.1. (continued)

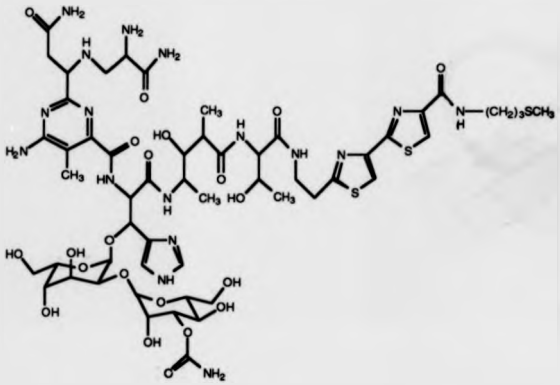
1.3.1. ANTIBIOTICS.

The first clinical use of an antibiotic, penicillin, was reported in 1941 (Abraham *et al.*, 1941) and until fairly recently, the main thrust of the search for new microbial products was still centered around the discovery of antimicrobial or antifungal agents. The large number of antibiotics found and amount of knowledge accumulated about them bears testament to the importance placed on them.

Antibiotic compounds produced by microorganisms show enormous structural diversity, ranging from small, low-molecular weight molecules such as methylenomycin A (Haneishi *et al.*, 1974) to large macrolides and peptide antibiotics such as bleomycin (Crandall and Hamill, 1986). These metabolites are shown in Figure 1.2.



Methylenomycin A



Bleomycin

Figure 1.2.

The usefulness of certain antibiotics in the treatment of infection lies in the fact that their target sites in the bacteria are not found in the host's cells. β-Lactam antibiotics (eg. penicillins and cephalosporins), for example interfere with the bacterial cell wall biosynthesis and aminoglycosides such as streptomycin and kanamycin act by binding to the 30S subunit of bacterial ribosomes to cause interference to protein biosynthesis. As the eukaryotic cells of the host contain a different type of ribosome and do not need to have peptidoglycan in their cell walls, they are unaffected by the antibiotic whilst the bacterium is killed. Despite the number of clinically useful antibiotics already available, the search for new ones is still important due to the development of resistance to commonly used antibiotics amongst previously treatable pathogens.

1.3.2. NON-ANTIBIOTIC MICROBIAL SECONDARY METABOLITES.

Although the discovery of new antibiotics is still important, the realisation that microorganisms may be the source of pharmacologically useful but non-antibiotic metabolites has led to a targeted search for such compounds. The search starts with the identification of a target for the screen. This may be a particular receptor or enzyme playing a key role in some stage of disease development, or a viral life

cycle. A screening system is then built around the target which needs to be selective, sensitive, high-throughput and preferably simple, cheap and amenable to automation. Some of the compounds shown up in such screens are already known antibiotics and some are new compounds which may or may not also show antibiotic activity. As mentioned before, these may be useful in their natural form, but may also serve as a starting point for the chemical synthesis of analogues for trial.

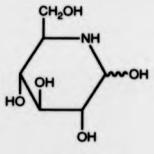
1.4. NITROGEN-CONTAINING GLYCOSIDASE INHIBITORS FROM PLANTS AND MICROORGANISMS.

1-Deoxynojirimycin (DNJ) is one of a group of nitrogen-containing compounds which have been isolated from plants and microorganisms over the last 30 years. They have been found to be potent inhibitors of glycosidases and have attracted much interest due to these and their other properties, including antiviral and antineoplastic activities.

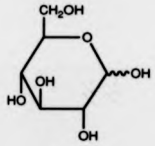
1.3.1. HISTORY AND ENZYME INHIBITORY PROPERTIES.

1.3.1.1. NOJIRIMYCIN.

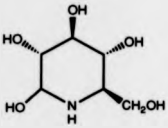
Nojirimycin, shown in Figure 1.3, (5-amino-5-deoxy-D-glucopyranose) was first described in 1966 (Inouye *et al.*, 1966) and has been isolated from fermentations of *Streptomyces lavendulae* SF425, *S.roseochromogenes* R468, *S.nojiriensis* SF426 (Ishida *et al.*, 1967a), *S.lavendulae* ATCC 31434 (Ezure *et al.*, 1985) and *S.ficellus* Dietz sp. NRRL8067 (Argoudelis and Reusser, 1976) as well as *Bacillus amyloliquefaciens* DSM7, *B.polymyxa* DSM365, *B.subtilis* DSM704 and *B.subtilis* var. *niger* DSM675 (Schmidt *et al.*, 1979). It was originally discovered on the basis of its antibiotic activity against a streptomycin and chloramphenicol resistant strain of *Shigella flexneri* (Nishikawa *et al.*, 1965). Nojirimycin was also shown to be able to prevent rice plant disease caused by *Xanthamonas oryzae* (Ishida *et al.*, 1967b).



Nojirimycin



Glucose



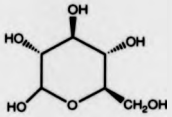


Figure 1.3.

Nojirimycin is a structural analogue of glucose, see Figure 1.3., and even shares some of its properties, for example, it will react with the enzymes glucose oxidase and glucose dehydrogenase (Muller, 1986) and will reduce Fehlings regent (Ishida *et al.*, 1967a). Nojirimycin is reported to be unstable in aqueous solutions at neutral or acidic pH which lead to problems with early attempts at isolation (Ishida *et al.*, 1967). However conversion to its more stable bisulphite adduct, shown in Figure 1.4, eases isolation and storage Nojirimycin has since been isolated more easily as its

bisulphite adduct (Argoudelis and Reusser, 1976; Inouye *et al.*, 1968).

Cyclic form Open form

Figure 1.4.

The first report of the enzyme inhibitory properties of nojirimycin came in 1970. Prompted by an observation by Cleayssens and DeBruyne that D-xylose derivatives with ring sulphur or nitrogen atoms were capable of inhibiting some glycosidases (Cleayssens and DeBruyne, 1965), Niwa *et al.* looked for similar traits in nojirimycin. They found that β-glucosidases from *Trichoderma viride* and apricot emulsin as well as Takadiastase and glucoamylase from *Rhizopus niveus* were inhibited by the nojirimycin (Niwa *et al.*, 1970). Reese *et al.* expanded on these findings by studying the inhibition of various glycosidases by nojirimycin and its bisulphite

adduct and found that exo-glucanases such as trehalase were less strongly inhibited than glucosidases were and that endo-glucanases including the α- and β-amylases were not inhibited at all (Reese *et al.*, 1971). The basis of the inhibition of these enzymes by nojirimycin lies in its resemblance to the glucose units in their substrates. The bisulphite adduct of nojirimycin is also able to inhibit glycosidases and has been shown to exist in a cyclic form (Kodama *et al.*, 1985) like nojirimycin so that it too resembles glucose. However, although both nojirimycin and its bisulphite adduct are potent inhibitors of β-glucosidases, nojirimycin has also been shown to inhibit α-glucosidase whereas the bisulphite adduct does so only very weakly (Kodama *et al.*, 1985). This may be because whereas nojirimycin exists as a 60:40 mixture of α-:β- epimers (Niwa *et al.*, 1970) , the bisulphite adduct only exists, in aqueous solution as the β-configuration (Kodama *et al.*, 1985), and so cannot bind to and inhibit the α-glucosidase. Studies on the affect of pH on the potency of the inhibition by nojirimycin indicate that the inhibitor binds in its unprotonated form (Dale *et al.*, 1985).

Other biological properties of nojirimycin include its ability to alter glycoprotein biosynthesis (Peyrieras *et al.*, 1983). In the presence of nojirimycin, a much shortened oligosaccharide chain is transferred to the glycosylation site of the protein (the biosynthesis of glycoproteins will be discussed in more detail later). Although the exact

mechanism is not known, nojirimycin itself may be transferred to the glycan and cap it so that only a truncated glycan is made (Peyieras *et al.*, 1983)

1.4.1.2. NOJIRIMYCIN B.

Nojirimycin B (5-amino-5-deoxy-mannopyranose), shown in figure 1.5, is an analogue of mannose in the same way that nojirimycin is an analogue of glucose. It has been co-isolated as its bisulphite adduct with nojirimycin from *Streptomyces lavendulae* SF425 and found to show weak antibiotic activity against *Xanthomonas oryzae* (Niwa *et al.*, 1984).

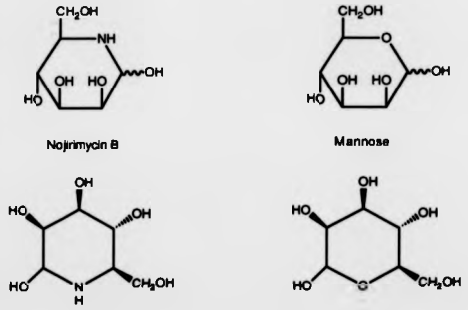


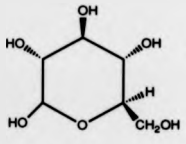
Figure 1.5.

Nojirimycin B inhibits emulsin β-glucosidase with a comparable potency to nojirimycin. Nojirimycin B bisulphite adduct has been shown to be a stronger inhibitor of this enzyme than either of the native nojirimycins (Niwa *et al.*, 1984).

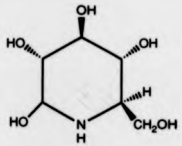
1.4.1.3. CASTANOSPERMINE.

Castanospermine, (1R,6S,7R,8R,8aR-tetrahydroxy-octahydroinolizine), was first isolated in 1981 from the seed of the legume *Castanospermum australe*, found in the rain forest of eastern Australia (Hohenschutz *et al.*, 1981) and has since been isolated from the dried pod of another legume, *Alexa leiopetala* Sandwith, which comes from Guyana and several other members of this genus (Nash *et al.*, 1988).

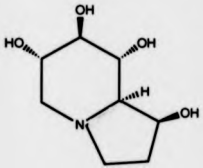
The six-membered ring of castanospermine has three hydroxyl groups which have the same stereochemistry as the corresponding ones in glucose and nojirimycin. The hydroxyl group at C-1 in glucose and nojirimycin is missing in castanospermine and the five membered ring acts like a bridge between the ring nitrogen and the C-6 of nojirimycin (see Figure 1.6).



Glucose



Nojirimycin



Castanospermine

Figure 1.6.

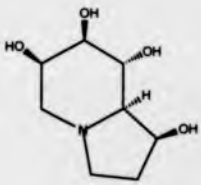
The enzyme inhibitory properties of castanospermine also resemble nojirimycin. It has been found to inhibit lysosomal α-glucosidase and β-glucosidases from almond emulsin a (Saul *et al.*, 1983) and the bruchid beetle (Fellows, 1987). Castanospermine also inhibits *Aspergillus* β-xylosidase, β-glucocerebrodase (Saul *et al.*, 1983), and a range of other α- and β-glucosidases including cockroach trehalase (Jahagirdar *et al.*, 1990), amyloglucosidase (Saul *et al.*,

1984) and glucosidase I involved in glycoprotein processing (Pan *et al.*, 1983, Hori *et al.*, 1984, Szumilo *et al.*, 1986a). Glucosidase II of glycoprotein processing is also inhibited by castanospermine, but to a lesser extent than glucosidase I (Pan *et al.*, 1983, Kaushal *et al.*, 1988).

Castanospermine has been identified as an antifeedant for aphids (Dreyer *et al.*, 1985). This may be due to its inhibition of thioglucosidase from mustard and aphids (Schofield *et al.*, 1990) which hydrolyses natural antifeedants produced by plants.

1.4.1.4 6-EPICASTANOSPERMINE.

6-Epicastanospermine (1S,6R,7R,8R,8aR-tetrahydroxy-octahydroindolizine), shown in Figure 1.7, has also been isolated from *Castanospermum australe*. Like castanospermine, this compound was found to strongly inhibit amyloglucosidase but unlike castanospermine it also showed some activity against β-galactosidase and only poor activity against β-glucosidase and the glucosidases I and II of glycoprotein processing. Common with castanospermine, no inhibitory activity was observed against either α- or β-mannosidases (Molyneux *et al.*, 1986).



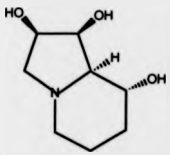
6-Epicastanospermine

Figure 1.7.

1.4.1.5. SWAINSONINE.

Swainsonine, (1S,2R,8R,8aR-trihydroxyloctahydroindolizine), shown in Figure 1.8, was first isolated in 1973 from the fungus *Rhizoctonia leguminicola* (Guengerich *et al.*, 1973), a known producer of a similar alkaloid, slaframine, which is known to cause excess salivation in cattle (Aust *et al.*, 1966). It has since been isolated from the Darling pea, *Swainsona canescens*, from Australia (Colegate *et al.*, 1979) and two types of locoweed found in the U.S.A., *Astragalus lentiginosus* (Molyneux and James, 1982) and *Astragalus emoryanus* (Davis *et al.*, 1984), all of which can cause poisoning of cattle. The symptoms of the poisoning are very similar to those of a condition found in man and other

mammals called mannosidosis, caused by a genetic deficiency in the mannosidase enzyme (Davis *et al.*, 1984). Swainsonine has also, more recently, been found in another fungus, *Metarhizum anisopliae* F3622 (Hino *et al.*, 1985a).

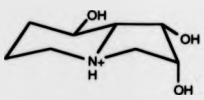


Swainsonine

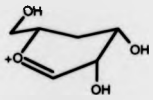
Figure 1.8.

Swainsonine has been shown to be the agent in *Swainsona* which is responsible for the cattle poisoning (Dorling *et al.*, 1980). The similarity between this poisoning and mannosidosis is due to the inhibition of mannosidases by swainsonine, a potent and selective inhibitor of α-mannosidases from jack bean and lysosomal preparations (Dorling *et al.*, 1980, Schneider *et al.*, 1983, Colegate *et al.*, 1979, Kang and Elbein, 1983a), and of the glycoprotein processing mannosidase II involved in the maturation of complex type glycans (Tulsiani *et al.*, 1982). Swainsonine is not an inhibitor of β-mannosidase, or α- and β-glucosidases (Kang

and Elbein, 1983a). The inhibition of mannosidase by swainsonine is competitive (Kang and Elbein, 1983a) involving the tight binding of the inhibitor, whose cationic form bears a strong resemblance to the mannosyl ion (Dorling *et al.*, 1980- see Figure 1.9) to the active site of the enzyme. Kinetic studies indicate that the inhibitor is removed, only slowly, from its binding site at high concentrations of substrate (Kang and Elbein, 1983a).



Swainsonine cation



Mannosyl cation

Figure 1.9.

1.4.1.6. DEOXYNOJIRIMYCIN (DNJ).

1-Deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol) was first isolated from a natural source in 1976 when it was found in the roots of the mulberry, *Morus sp.* (Yagi *et al.*, 1976). Prior to that date it had been synthesised through the catalytic hydrogenation of nojirimycin (Inouye *et al.*, 1968)

and since then it has been isolated from *Bacillus subtilis* (Schmidt *et al.*, 1979), *Streptomyces lavendulae* (Murao and Miyata, 1980, Ezure *et al.*, 1985) and another mulberry, *Morus bombylis* (Daigo *et al.*, 1986).

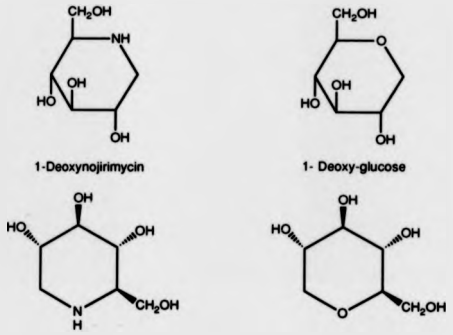
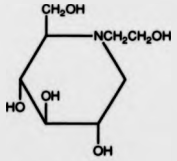


Figure 1.10.

DNJ is a structural analogue of 1-deoxy-glucose (see Figure 1.10) and it shows a similar range of enzyme inhibitory properties to nojirimycin. DNJ has been found to be a potent inhibitor of both α-and β-glucosidases, trehalase, β-

glucosidase (Murao and Miyata, 1980), aphid thioglucosidase (Schofield *et al.*, 1990), invertase and exo-β-1,3-glucanase. This inhibitor can also effect the production of glycoproteins by inhibiting the glucosidases I and II of glycoprotein processing (Szumilo *et al.*, 1986a, Saunier *et al.*, 1982).

The inhibition by DNJ of α-glucosidase, an enzyme which is responsible for the final steps of carbohydrate digestion, has led to hopes that it might be useful in the treatment of diabetes mellitus. An analogue of DNJ, BAY m 1099 (Figure 1.11), has been observed to inhibit the post-prandial rise in glucose levels in non-insulin dependent diabetics without any adverse side effects (Joubert *et al.*, 1986; Schnack *et al.*, 1986).



BAY m 1099

Figure 1.11.

1.4.1.7. DEOXYMANNOJIRIMYCIN (DMJ).

DMJ (1,5-dideoxy-1,5-imino-D-mannitol) has been isolated from the tropical legume *Lonchocarpus sericeus* in 1979 (Fellows *et al.*, 1979) and also from cultures of *Streptomyces lavendulae*, which also produces DNJ (Ezure *et al.*, 1988).

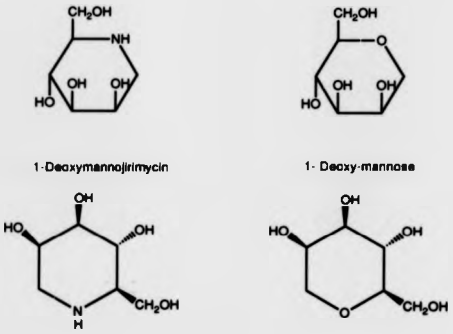


Figure 1.12.

DMJ is an analogue of 1-deoxy-mannose (see Figure 1.12) which is reflected in its inhibition of α-mannosidases including the α-mannosidases IA and IB of glycoprotein processing

(Fuhrmann *et al.*, 1985, Evans *et al.*, 1985), and also α-L-fucosidase (Winchester *et al.*, 1990). An early report that DMJ was an inhibitor of insect trehalase (Evans *et al.*, 1983) was later shown to be due to contamination with another inhibitor, DMDP (Evans *et al.*, 1985).

1.4.1.8. 2R,5R-DIHYDROXYMETHYL-3R,4R-DIHYDROXY-PYRROLIDINE (DMDP).

DMDP, shown in Figure 1.13, was initially isolated from *Derris elliptica* in 1976 (Welter et al., 1976) and later from another tropical legume, *Lonchocarpus sericeus*, which also produces DMJ (Evans *et al.*, 1985).

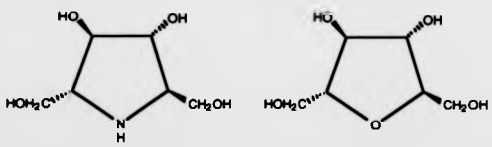


Figure 1.13.

Structurally, DMDP is analogous to fructose and, in common with other compounds already described, is an inhibitor of a range of glycosidases. DMDP will inhibit α- and β-glucosidases (Fleet and Fellows, 1988; Evans *et al.*, 1985) and others including trehalase, β-xylosidase, invertase (Evans *et al.*, 1985), maltase (Fellows, 1987), thioglucosidase (Schofield *et al.*, 1990) and α-mannosidases (Cenci di Bello *et al.*, 1985). DMDP also interferes with glycoprotein processing by inhibiting the action of trimming glucosidase I (Elbein *et al.*, 1984b) and has been found to act as an antifeedant against locusts, *Locusta migratoria* L. (Fleet and Fellows, 1988) and the bruchid beetle (Fellows, 1987).

1.4.1.9. OTHERS.

The compounds described above have received considerable interest due to their potentially useful biological activities, which will be discussed later, but they are not the only glycosidase inhibitors produced by plants or microorganisms which are structural analogues of monosaccharides. Others in this class include the *Streptomyces lydicus* product galactostatin (5-amino-5-deoxy-D-galactopyranose) which inhibits β-galactosidases from a

range of sources (Miyake and Ebata, 1987), fagomine (1,2,5-trideoxy-1,5-imino-D-arabinohexitol) from buckwheat, *Fagopyrum esculentum*, which inhibits isomaltase, BR1 (2S-carboxy-3R,4R,5S-trihydroxypiperidine) which is a β-glucuronidase inhibitor from the legume *Baphia racemosa*, AB1 (1,4-dideoxy-1,4-imino-D-arabinitol), the α-glucosidase inhibitor from another legume *Anglocalyx boutiqueanus* (Fleet and Fellows, 1988), and a third hydroxylated pyrrolizidine from *Castanospermum australe*, called australine ((1R,2R,3R,7S,7aR)-3-(hydroxymethyl)-1,2,7-trihydroxypyrrolizine), which is an inhibitor of amyloglucosidase and of glycoprotein processing glucosidase I (Tropea *et al.*, 1989). All of these compounds are shown in Figure 1.14.

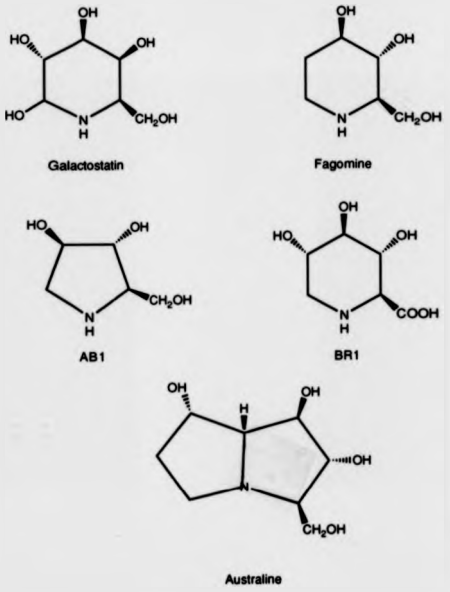

OH
HO
OH
HO
N
H
CH2OH
Galactostatin
OH
OH
N
H
CH2OH
Fagomine
HO
OH
N
H
CH2OH
AB1
OH
HO
OH
N
H
COOH
BR1
OH
H
OH
OH
N
CH2OH
Australine

Figure 1.14.

1.4.2. POTENTIAL MEDICAL AND SCIENTIFIC APPLICATIONS OF THE INHIBITORS.

Members of this family of glycosidase inhibitors are showing promise as tools in research in areas of biology and medicine. The specificity and potency of the inhibitors has led to their employment in studying the biological roles of the enzymes they inhibit and they have been especially useful in the study of the role of the glycan moieties of glycoproteins. The potential application of DNJ derivatives as antidiabetic agents has already been discussed and this and others of these inhibitors also showing promise as antiviral and antimetastatic agents.

1.4.2.1. BIOSYNTHESIS OF GLYCOPROTEIN GLYCANS.

The glycan moieties of glycoproteins are added co-translationally to asparagine residues in the polypeptide chain occurring in a specific sequence of four amino acids. The initial stage in the synthesis is the production of a lipid-linked oligosaccharide from which the $Glc_3Man_9NAG_2$ structure is transferred *en bloc* to the protein which is being threaded through the membrane of the rough endoplasmic reticulum into its lumen during its synthesis. The oligosaccharide then undergoes a series of trimming reactions

to produce a finished glycan of either the complex or high-mannose type according to the glycoprotein being produced. These trimming reactions are shown in Figure 1.15.

The first enzymes to act on the structure are located in the rough endoplasmic reticulum membrane and are the trimming glucosidases I and II. Glucosidase I removes the terminal α-1,2-linked glucose residue from the structure and glucosidase II sequentially removes the next two α-1,3-linked ones. There is some evidence that a third glucosidase may also be capable of removing the third glucose (Fuhrmann *et al.*, 1985). At this stage the glycan may stay as a high mannose type or undergo further processing which begins with the removal of four α-1,2- linked mannose residues. The first can be removed by an α-mannosidase in the endoplasmic reticulum but the rest of the processing takes place in the golgi where mannosidases 1A and 1B are situated and are both capable of removing all four of the residues. Following the addition to the structure of an N-acetylglucosamine (NAG) unit, a further two mannoses are removed by the action of mannosidase II. A series of glycosyl transferases then elaborate the structure with galactose, sialic acid, NAG and fucose residues to produce the final complex glycan. Once the trimming reactions are complete, the glycoprotein leaves the golgi and is transported to its site of action eg. on the surface of the cell or excreted from it.

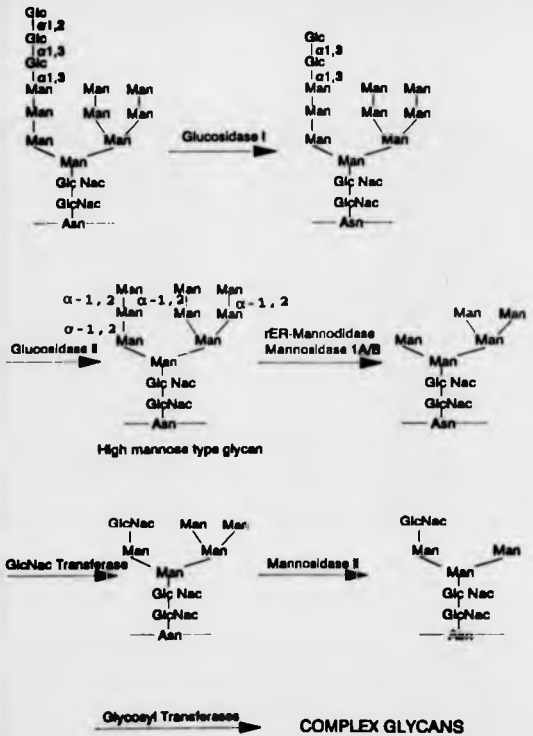


Figure 1.15. Trimming reactions of glycoprotein biosynthesis.

The use of inhibitors of glycoprotein processing including DNJ, DMJ, swainsonine and castanospermine allow the specific modification of glycans so that the role of the glycan or individual parts of it can be studied have shown that the glycans of glycoproteins have a variety of roles. They can act to protect the protein from proteolysis, direct the cleavage of a larger precursor glycoprotein, modify the physical or biological properties of the protein, direct the protein to its correct location in the cell, and have also been shown to play a part in embryonic development, cell-cell interactions and differentiation (Olden *et al.*, 1985). Glycans on different proteins may have different roles and the glycan moiety of a single glycoprotein may perform several functions.

The effect that these inhibitors have on the processing of glycoproteins is responsible for the antiviral activity shown by some of them.

1.4.2.2. ANTIVIRAL PROPERTIES.

In 1987, the first reports of DNJ and castanospermine acting as antiviral agents appeared. Three groups (Walker *et al.*, 1987; Tyms *et al.*, 1987; Gruters *et al.*, 1987) reported activity against Human Immunodeficiency Virus (HIV), the causative agent in AIDS and a fourth reported their activity against another retrovirus, and HIV model, Moloney leukemia

virus (Sunkara *et al.*, 1987). DMDP was also initially reported to show activity against HIV replication (Tyms *et al.*, 1987) but the inhibition of replication was later shown to be inseparable from the cytotoxicity of the compound (Karpas *et al.*, 1988). In the first reports, DNJ and castanospermine were shown to inhibit the production of multinucleated cells, or syncitia, produced by the fusion of cells carrying the CD4 antigen in the presence of HIV or HIV infected cells in a process similar to that of the entry of the virus to the cell. They were also shown to reduce viral infectivity at non-cytotoxic concentrations. It is believed that their antiviral activity comes from their ability to alter the glycosylation of the HIV envelope glycoprotein gp120. Gp120 has 24 potential sites for N-glycosylation (glycosylation through an asparagine residue, as described earlier) and the glycans make up 50% of its molecular weight (Karpas *et al.*, 1988). Most of the glycans on gp120 exist as the high-mannose type (Shimizu *et al.*, 1990).

The initial step in the infective cycle of HIV is the interaction between gp120 and the CD4 antigen on the surface of some cells of the immune system. This leads to the internalisation of the virus by either endocytosis or fusion of the viral envelope and the cell membrane (Grewe *et al.*, 1990). The inhibition of syncitium formation by DNJ and castanospermine show that this interaction and fusion process is interfered with by these compounds. Gp120 made in the presence of DNJ or castanospermine will have the three

glucose residues normally removed by trimming glucosidases I and II still present. The terminal glucose residue appears to be the crucial one as bromoconduritol, an inhibitor which only inhibits glucosidase II, is inactive against HIV (Sunkara *et al.*, 1987). The abnormal gp120 produced in the presence of DNJ or castanospermine has been found to have a comparable affinity for CD4 to gp120 to its normal glycan (Walker *et al.*, 1987) so latter stages of the interaction such as the fusion of the membranes are affected by the change in the glycan, and not the initial binding to the receptor. It has recently been proposed that an alteration in the conformation of the V3 loop of gp120 is induced by the alteration in its glycosylation. Cleavage of the V3 loop necessary for infection of the cell after gp120-CD4 binding may be inhibited by this conformational change (Jones and Jacob, 1991). Reduced cleavage of the gp120 and gp41 precursor, gp160 (Walker *et al.*, 1987; Pal *et al.*, 1989) and reduced surface expression of gp120 (Walker *et al.*, 1987) as is also seen with other surface glycoproteins in the presence of these inhibitors (Edwards *et al.*, 1989) may also contribute to the reduction of infectivity of the virus.

Since the discovery that castanospermine and DNJ are capable of reducing the infectivity of HIV, the synthesis of analogues with increased activity and reduced toxicity has been a major target. In 1988, a series of N-alkylated DNJ molecules were produced by Fleet *et al.* and tested against the virus (Fleet *et al.*, 1988; Karpas *et al.*, 1988). Of

these, N-methyl DNJ, N-ethyl DNJ and N-butyl DNJ, all shown in Figure 1.16, were found to be particularly potent, reducing syncitium formation by 100% at non-cytotoxic concentrations (Fleet *et al.*,1988). Furthermore, prolonged incubation of HIV infected cells with N-butyl DNJ was found to result in the levels of the virus being reduced to undetectable levels and perhaps being eliminated from the cell culture (Karpas *et al.*, 1988). N-butyl DNJ is undergoing clinical trials in the U.S.A. for possible use as an anti HIV drug.

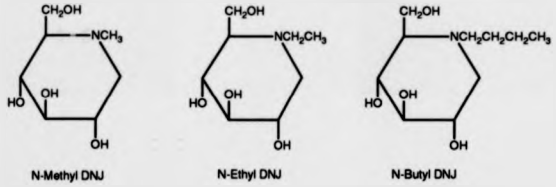


Figure 1.16.

In 1990, Shimizu *et al.* (Shimizu *et al.*, 1990) reported the synthesis of some more DNJ derivatives, of which some were even more potent reducers of syncitium formation than N-butyl DNJ. These compounds showed no cytotoxicity at concentrations used and are shown in Figure 1.17.

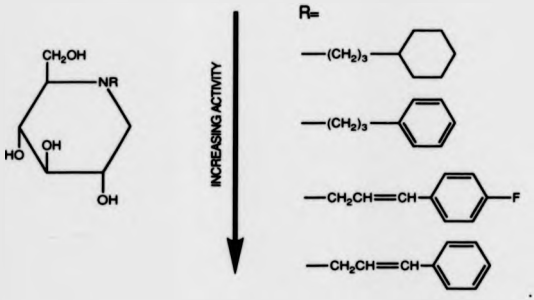


Figure 1.17.

Although various inhibitors have been found to alter the pattern of glycosylation of surface glycoproteins of the influenza virus (Elbein *et al.*, 1984a; Pan *et al.*, 1983), the vesicular stomatitis virus (Kang and Elbein, 1983b), Semliki forest virus, (Naim and Koblet, 1988), and fowl plague virus

(Romero *et al.*, 1983), the infectivity of each of these viruses remained unaffected by the changes. Castanospermine has, however, also been reported to reduce the infectivity of cytomegalovirus, as have DNJ and DMDP (Taylor *et al.*, 1988), and prolong the survival of mice infected with Rauscher Murine Leukemia virus, though it was less effective and more toxic than AZT (Ruprecht *et al.*, 1989). Glucosidase inhibitors also reduce the infectivity of Feline Immunodeficiency Virus (Stephens *et al.*, 1991).

1.4.2.3. ANTINEOPLASTIC AND IMMUNOMODULATORY PROPERTIES.

The oligosaccharide components of cell surface glycoproteins have been found to be altered from their normal forms when a cell becomes cancerous. In general, this alteration involves a change to a more highly branched and sialylated structure (Ogata *et al.*, 1976; Pierce and Arango, 1986) and evidence suggests (Kerbel *et al.*, 1987) that it may be involved in promoting the fast rate of growth in associated with cancer cells and later metastasis. Metastasis of cancers, the removal of cells from a primary tumor and their movement and colonisation of a secondary site, is the major cause of death in cancer patients and is much increased by surgery (Olden *et al.*, 1988).

These observations led to the testing of the effect of glycoprotein processing inhibitors on tumor growth and metastasis. In 1985, inhibition of pulmonary metastasis of B16 melanoma and growth of sarcoma 180 ascites tumor in mice by swainsonine was reported (Hino *et al.*, 1985b) and a year later, both swainsonine (Humphries *et al.*, 1986a) and castanospermine (Humphries *et al.*, 1986b) were shown to inhibit the experimental metastasis of B16-F10 murine melanoma cells. They have also been shown to inhibit the proliferation of tumor cells (Hadwiger *et al.*, 1986; Dennis *et al.*, 1990). The anticancer effects of these compounds have been attributed to changes in the structure of glycoprotein glycans, in particular the loss of β-1-6 branched complex type glycans (Yagel *et al.*, 1989; Dennis *et al.*, 1990). Alterations in the immunogenic properties of the altered glycoproteins may at least partly explain these properties of swainsonine and castanospermine. The antiproliferative effect of interferon was demonstrated to be enhanced by swainsonine treatment (Dennis, 1986) and later, glycosylation changes induced by swainsonine were found to make cancer cells more susceptible to interferon boosted natural killer cells (Ahrens and Ankel, 1987). The production of immunoglobulins (Karasuno *et al.*, 1990) and activities of interleukin-1, a stimulator of many immune responses, and tumor necrosis factor (Muchmore *et al.*, 1990) are also enhanced by changes in the oligosaccharides brought about by swainsonine. Also, an immunosuppression, caused by a protein factor, is often

associated with cancer and was found to be reversed by swainsonine both *in vitro* and *in vivo* (Hino *et al.*, 1985a; Hino *et al.*, 1985b).

Inhibition of metastasis of B16-BL6 melanoma and M5076 reticulum sarcoma tumor cells is not limited to experimentally simulated metastasis (Newton *et al.*, 1989) but different types of metastatic cell lines are affected differently with respect to their ability to metastasise by different inhibitors of glycoprotein processing (Bruyneel *et al.*, 1990). This indicates that although at this point only swainsonine and castanospermine are well characterised as inhibitors of cancer growth and metastasis in the relatively small sample of cancer cell lines studied so far, others in the group may yet be found to show similar effects in other types of cancers.

OBJECTIVES.

The aims of this project were to achieve a greater understanding of the biosynthesis of 1-deoxynojirimycin (DNJ) in *Streptomyces*.

One aim was to develop a more selective enzymatic assay system for DNJ than the ones used to date (eg. Stein *et al.*, 1984; Ezure *et al.*, 1985), and to use this to screen for new producers and study the biosynthesis of DNJ in these

organisms and in the known producer, *Streptomyces lavendulae* ATCC 31434.

The effects of different culture conditions and nutrients were also investigated in order to assess factors which play an important role in the regulation of DNJ biosynthesis.

The biosynthetic pathway of DNJ was also studied with the use of isotopically labeled precursor molecules.

CHAPTER 2 ASSAY AND SCREENING.

2.1. INTRODUCTION.

METHODS FOR SCREENING AND ASSAYING MICROBIAL METABOLITES.

The importance of a good assay in the study of the production of a secondary metabolite cannot be over emphasised. An assay should be sensitive, selective (ideally for a single metabolite), accurate, and, especially when many samples are to be assayed, preferably quick and inexpensive. The initial interest in a particular metabolite may arise from a positive hit in a screen and as the requirements for a good screening procedure are essentially the same as those for a quantitative assay, the former may be adapted to allow quantification of the metabolite. Although screening for a product can be carried out without any knowledge of the structure of the metabolite concerned, its quantification requires the purification of a sample for use as a standard in an assay.

Screening and assay methods can be grouped into a number of different classes, the most traditional of these being the antimicrobial bioassay. The antibiotic activity of many secondary metabolites makes them amenable to study by this

kind of system which involves the use of a microorganism which is sensitive, or hypersensitive, to the compound concerned without being affected by other compounds made by the organism under investigation. Quantification may be carried out using the agar diffusion method, for example. This involves unknown samples as well as standard solutions of known concentrations being used to impregnate discs of paper. These are then laid on the top of an agar plate seeded with the sensitive organism or are put directly into wells cut in the agar. The zone size of non-growth around the well or disc can be used to establish the antibiotic titre by comparison to standards of known concentration. This method is used in the assay of many antibiotics.

Many secondary metabolites are capable of acting as enzyme inhibitors and can be assayed on the basis of this activity. The choice of enzyme is important where a compound is capable of inhibiting more than one. It must be inhibited strongly enough to provide a sensitive assay and should be selective for only the inhibitor of interest. The production of families of metabolites with similar chemical structures can lead to problems with selectivity as the biological activity of the compounds might also be similar. Finding an enzyme inhibited by only one of these can be difficult.

The problem of selectivity can be overcome by the use of combined separation and assay methods such as High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC), which are also used when the target

compound has no utilisable biological activity. These assay techniques require the development of a suitable separation protocol and in the case of metabolites which are not easily detected may require a derivatisation procedure to attach fluorescent or U.V. active chromophores prior to assay.

ASSAY AND SCREENING METHODS FOR DEOXYNOJIRIMYCIN (DNJ).

Assay methods for DNJ in microbial fermentations need to be selective as both nojirimycin (NJ) (Ezure *et al.*, 1985; Stein *et al.*, 1984) and deoxymannojirimycin (DMJ) (Ezure *et al.*, 1988) may be produced simultaneously by organisms. Glycosidase inhibitory properties provide a convenient method for the assay of these compounds, but although the enzyme inhibitory spectrum of DMJ is significantly different from that of DNJ, that of NJ is very similar (Table 2.1.), which poses problems when attempting to quantify DNJ without interference from NJ. Quantitative assays for DNJ have accounted for this by assaying the two compounds using enzymes which are strongly inhibited by one inhibitor and less so by the other. The use of Rabbit intestinal sucrase and emulsin β-glucosidase to assay DNJ and NJ respectively in microbial fermentations has been reported (Ezure *et al.*, 1985; Stein *et al.*, 1984). Both of these enzymes are inhibited by both DNJ and NJ. The β-glucosidase is inhibited

ENZYME	SOURCE	REFERENCE
Sucrase	Aureobasidium pullulans	Reese et al., 1971
	Saccharomyces sp.	Reese et al., 1971
	Rabbit small intestinal	Hanozet et al., 1981
Glucoamylase	not given	Schmidt et al., 1979
	Aspergillus niger *	Reese et al., 1971
	Endomyces sp. *	Reese et al., 1971
	Rhizopus niveus	Niwa et al., 1970
β-Glucosidase	Emulsin *	Evans et al., 1983
	Almond emulsin *	Reese et al., 1971
	Aspergillus luchuensis *	Reese et al., 1971
	Aspergillus niger *	Reese et al., 1971
	Aureobasidium pullulans *	Reese et al., 1971
	Penicillium miliniI *	Reese et al., 1971
	Apricot emulsin	Niwa et al., 1970
	Trichoderma viride	Niwa et al., 1970
α-Glucosidase	Yeast *	Evans et al., 1983
	Aspergillus niger *	Reese et al., 1971
	Trichoderma viride *	Reese et al.,1971
	Penicillium parvum	Reese et al., 1971
	Paecilomyces varioti*	Reese et al., 1971
Trehalase	Trichoderma reesei	Alabran et al., 1983
	Aureobasidium pullulans	Reese et al.,1971
	Penicillium parvum	Reese et al., 1971
Myrosinase	Yellow mustard	Reese et al., 1971
	Aspergillus oryzae	Reese et al., 1971
Exo-β-1,3-glucanase	Basidiomyces sp. *	Reese et al., 1971
	Chrysosporium pruinosum *	Reese et al., 1971
Maltase	not given	Schmidt et al., 1979
Cellulase	Basidiomyces sp.	Reese et al., 1971
	Chrysosporium pruinosum	Reese et al., 1971
	Trichoderma viride	Reese et al., 1971
α-Amylase	not given	Niwa et al., 1970
β-Amylase	not given	Niwa et al., 1970
α-Mannosidase	Rat epididymus	Kodama et al., 1986
β-Mannosidase	Penicillium spp.	Reese et al., 1971
	Snail	Reese et al., 1971
Isomaltase	not given	Schmidt et al., 1979
β-Galactosidase	Penicillium miliniI	Reese et al., 1971
Takadiastase	Rhizopus niveus	Niwa et al., 1970

* = inhibitor used as bisulphite adduct

Table 2.1. Glycosidases inhibited by NJ.

GLYCOSIDASES INHIBITED BY DEOXYMANNOJIRIMYCIN

ENZYME	SOURCE	REFERENCE
α-Mannosidase	Jack bean	Evans et al., 1985
	Human liver	Winchester et al.,1990
α-Fucosidase	Bovine epididymus	Evans et al., 1985
	Human liver	Winchester et al., 1990
Mannosidase I	Mung beans	Szumilo et al., 1986b
	Rat liver	Bischoff and Kornfeld, 1984
Glucosidase I	Calf liver	Schweden et al., 1986

GLYCOSIDASES INHIBITED BY DEOXYNOJIRIMYCIN

ENZYME	SOURCE	REFERENCE
α-Glucosidase	Yeast	Evans et al., 1985
		Murao and Miyata, 1980
	Rabbit	Murao and Miyata, 1980
ß-Glucosidase	Emulsin	Evans et al., 1985
	Almond	Murao and Miyata, 1980
		Dale et al., 1985
	Aspergillus aculeatus	Murao and Miyata, 1980
Trehalase	Chaetomium aureum	Murao and Miyata, 1980
	Rabbit	Murao and Miyata, 1980
	Cockroach	Nishimura et al., 1990
Glucosidase I	Calf liver	Schweden et al., 1986
		Fuhrmann et al., 1985
	Mung bean	Szumilo et al.,1986a
	Saccharomyces cerevisiae	Fuhrmann et al., 1985
Glucosidase II	S. cerevisiae	Fuhrmann et al., 1985
	Calf	Fuhrmann et al., 1985
α-1,6-Glucosidase	Rat liver	Bollen and Stalmans, 1986
Glucoamylase	Rhizopus niveus	Murao and Miyata, 1980
Thioglucosidase	Brevicoryne brassicae	Schofield et al., 1990
ß-Xylosidase	Aspergillus niger	Evans et al., 1985
Sucrase	Rabbit	Murao and Miyata, 1980
	Rabbit small intestine	Hanozet et al., 1981

Table 2.1.(Continued) Glycosidases inhibited by DNJ and DMJ

by NJ with an IC_{50} of 2.85×10^{-6}M and DNJ with one of 8.1×10^{-5}M (Schmidt *et al.*, 1979) and the sucrase with Ki values of 2.2×10^{-8}M and 1.2×10^{-7}M by DNJ and NJ respectively at the pH used by Ezure (Hanozet *et al.*, 1981). Although the two inhibitors show different strengths of inhibition of the two enzymes, both assays will become inaccurate in the presence of significant quantities of the other inhibitor.

In addition to the enzymatic assay for DNJ, Ezure also used an HPLC method involving separation on a reverse phase HPLC column and detection with a refractive index detector. A method for DNJ assay using GC has also been described by Fleet and Fellows (1988).

On the screening side, DNJ has been screened for using a plate screen based on the inhibition of glucoamylase catalysed degradation of starch in the presence of DNJ (Ezure *et al.*, 1985). DNJ has also been found in a *Streptomyces* strain during a screen for inhibitors of the trehalase of *Chaetomium aureum* MS-27 (Murao and Miyata, 1980) but no mention of its use in the quantification of DNJ has been reported.

2.2. MATERIALS AND METHODS.

2.2.1. STANDARD ASSAY CONDITIONS.

The trehalase assay used was based on that of Dahlqvist (Dahlqvist, 1984). The amount of glucose liberated from trehalose by trehalase was compared with and without the presence of inhibitor. The inhibitor was in the form of an aqueous solution of a standard inhibitor or a supernatant sample from a centrifuged microbial culture. Three incubations for each sample to be assayed were set up as follows.

	Blank	-Inhibitor	+Inhibitor
Trehalase solution in 0.1M maleate buffer pH 6.0 (0.0358 U\ml)	-	20µl	20µl
Inhibitor solution	20µl	-	20µl
Distilled water	20µl	20µl	-

These were incubated at 37°C for 15 minutes and then 56mM trehalose in 0.1M maleate buffer pH 6.0 (20µl) was added to each one to initiate the reaction. After a further incubation of 60 minutes at 37°C, the following additions were made.

Glucose assay kit solution (GOD-PAP from Boehringer)	1000µl	1000µl	1000µl
Inhibitor solution	-	20µl	-
Distilled water	20µl	-	20µl

A final incubation of 30 minutes at 37°C was followed by the reading of the absorbances of each solution at 510nm on a Pye-Unicam SP1800 UV spectrophotometer.

The values for the absorbances at 510nm with and without the inhibitor were corrected for the blank value and the percentage inhibition of trehalase by samples was calculated according to the equation below;

$$\% \text{ Inhibition} = 1 - \frac{A(510nm) + \text{Inhibitor}}{A(510nm) - \text{Inhibitor}} \times 100$$

2.2.2. SCREENING.

MAINTAINANCE OF MICROORGANISMS.

Strains of *Streptomyces* and *Streptoverticillium* were maintained on Oatmeal agar (see Appendix) plates at 4°C and in 20% glycerol at -20°C. Strains used regularly were sub-cultured at least monthly.

CULTIVATION OF MICROORGANISMS

Soyabean medium (Ezure *et al.*, 1985), consisting of 2% soluble starch, 1% soyabean meal, 0.05% KCl, 0.05% $MgSO_4$, $7H_2O$, 0.2% $NaNO_3$, 0.5% NaCl, and 0.35% $CaCO_3$ (pH 7.4) in 30ml aliquots in 250ml conical flasks were inoculated from freshly grown, and sporulating, *Streptomyces* or *Streptoverticillium* strains on oatmeal agar plates (see Appendix). They were then incubated at 28°C, 190 rpm, for 7 days after which the cultures were decanted into sterile Universal bottles and centrifuged in a Beckman JA20 rotor (3000 rpm, 10 min). The supernatant was decanted for assay.

TREHALASE SCREEN.

Supernatant samples were used under standard assay conditions without pre-treatment.

LARGER SCALE FERMENTATION, PURIFICATION AND ANALYSIS OF DNJ.

Strains giving inhibition in the trehalase screen were then grown on a 1l scale (25 x 40ml fermentations in soyabean medium). These were inoculated and incubated as for the original screening, pooled and centrifuged (3000rpm for 10 min.) in a Beckman JA20 rotor. The strong cation exchange resin, Dowex 50-X8 (H^+ form) (150g) was then added to the supernatant and this was stirred at room temperature for 1h. The resin was then removed by filtration and washed with water before being resuspended in 1M NH_4OH (500ml) and stirred for a further 1h at room temperature. The resin was again removed by filtration, washed with fresh 1M NH_4OH (200ml). The washings were pooled, evaporated to dryness under reduced pressure and then the residue resuspended in 70% aqueous ethanol (10ml). This solution was applied to an alumina column (100g) equilibrated in 70% ethanol and eluted with the same solvent. Samples of the 10ml fractions collected were subjected to T.L.C. analysis on silica plates using a butan-1-ol:ethyl acetate:acetic acid:water (1:1:1:1) solvent system and visualised with ninhydrin. Fractions

containing a component running with the same rf (0.42) as the DNJ standard were pooled and evaporated to dryness under reduced pressure. The residue was taken up in distilled water (5ml) and a sample (0.5ml) taken for mass spectrometric and gas chromatographic analysis.
The remainder from strain 445 and the patented producer ATCC 31434 were further purified for NMR analysis by preparative TLC using the same solvent system as above. The sample removed from the TLC plate was freeze dried and redissolved in deuterium oxide (2ml) for NMR analysis on a Bruker Spectrospin WH400 N.M.R..

GAS CHROMATOGRAPHIC ANALYSIS.

The sample (300µl) was freeze dried and then silylated with the addition of Sigma Sil-A (40µl) and incubation at 70°C for 20 minutes before injection into the chromatograph.
Sample 445 was run in a Pye-Unicam series 204 chromatograph fitted with a 5 ft glass OV-17 column. The gas flow (N_2) was 30ml\min, with a detector and injector temperature of 250°C and an oven temperature program of 125°C(2min) 6 \min 200°C (10 min).

Sample 194 was run in a Carlo Erba Fractovap 2450 chromatograph fitted with a 30m DB5 capillary column.

Detector\injector temperature was 250°C, the column was isothermal at 200°C and the N_2 flow was kept at 4ml\min.

2.2.3. USE OF TREHALASE INHIBITION TO QUANTIFY DNJ.

INHIBITION OF TREHALASE BY DEOXYNOJIRIMYCIN AND OTHER INHIBITORS.

The standard assay conditions described above were used to assess the degree of inhibition of trehalase by aqueous solutions of 1-deoxynojirimycin (DNJ), 1-deoxymannojirimycin (DMJ) and nojirimycin (NJ). The DMJ and DNJ used were obtained from Sigma and the NJ was obtained as its bisulphite adduct (gift of Prof. S. Ogawa, Keio University, Japan). Conversion to the free base was by the method of Inouye (Inouye *et al.*, 1968).

KINETICS OF THE INHIBITION OF TREHALASE BY DNJ AND NJ.

0.0385 U\ml trehalase in 0.1M maleate buffer pH 6.0 (20µl) were added to inhibitor solution (20µl), in triplicate, for each concentration of inhibitor and substrate used. These were incubated at 37°C for 15 minutes and then trehalose in 0.1M maleate buffer pH 6.0 (20µl) added. The three reactions were terminated by the addition of glucose assay kit solution (GOD-PAP from Boehringer) (1000µl) at 5, 10, and 15 minutes after the addition of the trehalose. Trehalose solutions used were 0, 14, 28, 42 and 56mM and at least two different inhibitor solution concentrations were used.

EFFECTS OF pH ON THE TREHALASE ASSAY.

Standard assay conditions were used with the inhibitor solution being 0.1mg\ml DNJ and the pH of the maleate buffer being varied between 5.5 and 7.0.

EFFECTS OF GLUCOSE CONCENTRATION ON THE TREHALASE ASSAY.

Standard assay conditions were used but with the inhibitor solution being replaced by aqueous solutions of D-glucose of concentrations up to 2mg\ml.

ELIMINATION OF INHIBITION OF TREHALASE DUE TO NJ.

Solutions of DNJ and NJ (100µl) were incubated with equal volumes of hydrochloric acid solutions for various lengths of time at different temperatures. They were then freeze dried and the residue resuspended in distilled water (100µl). The inhibition of trehalase due to these solutions was then assayed under standard assay conditions.

2.2.4. COMPARISON OF ASSAY OF DNJ BY TREHALASE METHOD TO THAT BY GAS CHROMATOGRAPHY.

Three samples were assayed by the two methods to compare the results. The first was an aqueous 0.05mg\ml solution of DNJ and the second was made by freeze drying 0.05mg\ml DNJ (0.5ml) and resuspending the residue in supernatant (0.5ml)

from a centrifuged culture of the non-DNJ producing *S.lividans* TK24 grown in soyabean medium. The third sample was a supernatant sample from a culture of the producer *S.subrutilus* 445.

HEAT\ACID TREATMENT OF SAMPLES.

The sample to be treated (200µl) was added to an equal volume of 6M HCl and heated in a boiling water bath for 6 hours. It was then freeze-dried and the residue resuspended in distilled water (200µl).

TREHALASE ASSAY.

The assay was carried out under standard conditions, in triplicate. DNJ concentrations were calculated by reference to the standard DNJ\trehalase inhibition curve.

GAS CHROMATOGRAPHIC ASSAY.

0.05mg\ml Methyl-β-D-glucoside (50µl) was added to the test solution (50µl) and then freeze-dried in a glass vial. The silylating agent Sigma Sil-A (50µl) was then added through a self sealing lid and the vial heated to 70°C for 20 minutes. The samples were then injected into a Carlo Erba Fractovap series 2450 preparative gas chromatograph fitted with a 30m J and W DB5 capillary column. The nitrogen flow rate was 3ml\min, the oven temperature an isothermal 190°C and the injector\detector temperature 250°C. The peaks were integrated using a Pye-Unicam DP88 computing integrator and the concentration of DNJ was calculated using the methyl-β-D glucoside peak as a standard. Each sample was assayed in triplicate.

2.3. RESULTS AND DISCUSSION.

2.3.1 SCREENING.

TREHALASE SCREEN.

Attempts to use a plate method for the screening of fermentations for DNJ based around its inhibition of glucoamylase (Ezure *et al.*, 1985) were unsuccessful, as an

STRAIN NUMBER	INHIBITION ? (% INHIBITION)
069	-
194	+ (15%, 16%)
316	-
216	-
094	-
190	-
550	-
445	+ (47%, 66%)
085	-
091	-
0255	-
0446	-
0500	-
0517	-
0524	-
0716	-
0936	-
184	-
191	-
233	-
260	-
422	-
454	-
512	-
620	-
630	-
742	-
639	-
675	-
642	-
7601	-
730	-
6.720	-
3602	-
146 RB	-
TK24	-
31434	+ (79%, 67%)
0767	-
0547	-
051	-

Table 2.2. Screening results.

unacceptable level of false positives were observed. For this reason an assay similar to one already described (Murao and Miyata, 1980) was adopted. The screening results are shown in Table 2.2.

PURIFICATION AND IDENTIFICATION OF DNJ FROM SCREENING POSITIVES.

The purification method used is based on the one used by Murao and Miyata (Murao and Miyata, 1980). The active charcoal step was omitted as was the anion exchange section. It was found that the coloration of the medium was removed efficiently by the alumina column and so the charcoal step was not needed and that the ommission of the anion exchange column did not affect the purity of the final product. Thin layer chromatography showed that the after the alumina column almost only DNJ and DMJ were left. Some, but not complete, separation of these two compounds was also seen in the fractions taken from this column.

The mass spectrum for 31434 (Fig. 2.1) shows a large peak at m\z=164, corresponding to M+1 for DNJ or DMJ. Other large peaks appear at m\z= 146, 132, and 110 are the results of successive dehydrations of the parent ion. The spectra of 194 (Fig. 2.2) and 445 (Fig. 2.3) both show the m\z=164 peak described above although the peaks for the fragment ions are lost in the other peaks caused probably by impurities in the

sample. These spectra also contain peaks at m\z=180 which might be due to NJ. However rapid dehydration of the NJ would make the appearance of this peak unlikely. Again the spectrum of 194 shows up more impurities than that of 445.

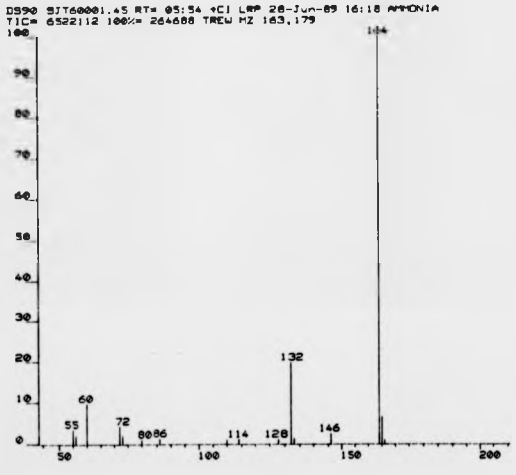


Figure 2.1. Mass spectrum of S.*lavendulae* 31434 product.

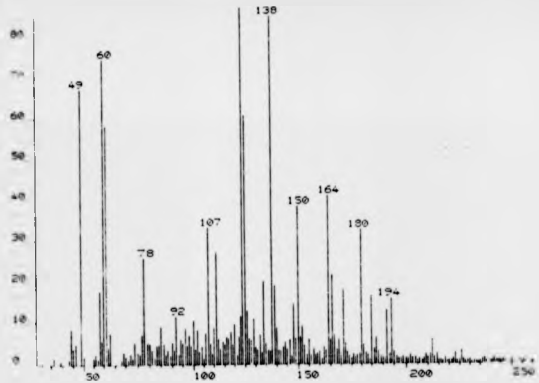


Figure 2.2. Mass spectrum of *S.racemochromogenes* 194 product.

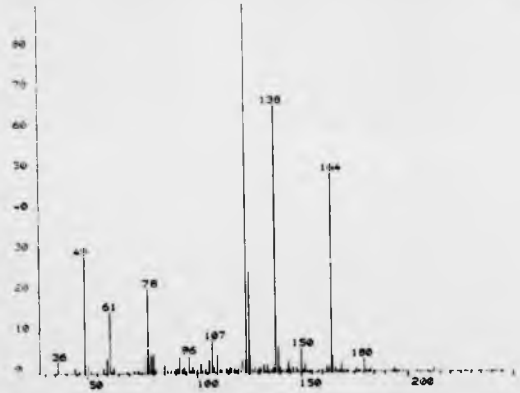


Figure 2.3. Mass spectrum of *S.subrutilus* 445 product.

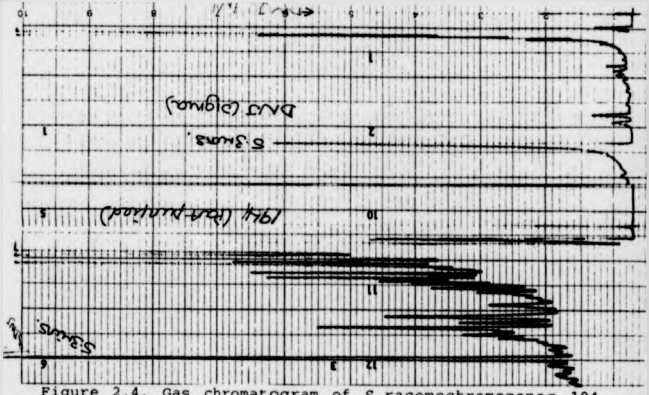


Figure 2.4. Gas chromatogram of *S.racemochromogenes* 194 product.

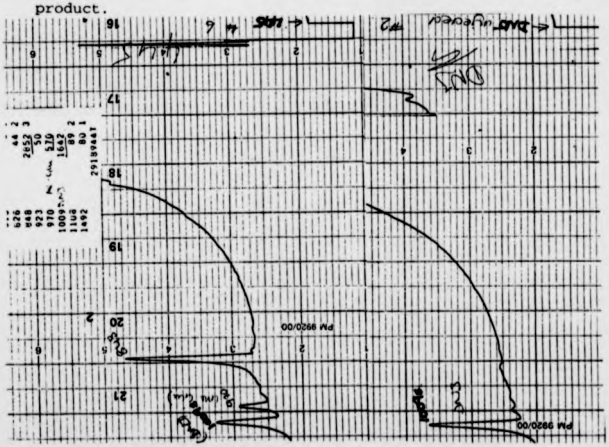


Figure 2.5. Gas chromatogram of *S.subrutilus* 445 product.

Gas chromatographic analysis of the partly purified fractions show the presence of DNJ in both 194 (Fig. 2.4) and 445 (Fig. 2.5) fermentations. The 445 sample shows only one main peak whereas the 194 sample has many. The GC method used for the 194 sample was more sensitive than for 445 implying that there was less DNJ in the 194 sample relative to the impurities. This coupled with the lower level of inhibition of trehalase shown by 194 indicate a smaller titre of DNJ in the 194 fermentation. For this reason it was decided to concentrate on strain 445 for further studies.

The final step of purification by preparative TLC allowed a little separation of the DNJ and DMJ although the degree of separation was variable. TLC of the final 31434 and 445 samples showed the presence of both DNJ and DMJ in each, although much less DMJ was present relative to DNJ in the 31434 sample. *S.lavendulae* has been shown to produce both compounds and these results show that 445 (*S. subrutilus*) does too. The different proportions of the two epimers in the samples from the two strains need not reflect differences in the production of these compounds by the two organisms as the degree of separation of DNJ and DMJ may vary in different separations. The isolation of DMJ from *Lonchocarpus sp.* (Fellows *et al.*, 1979) follows a similar route to that used here for DNJ. Recrystallisations of DNJ and DMJ from methanol (Ezure *et al.*, 1985; Ezure *et al.*, 1988) and methanol:acetone (Fellows *et al.*, 1979) have been used in purification

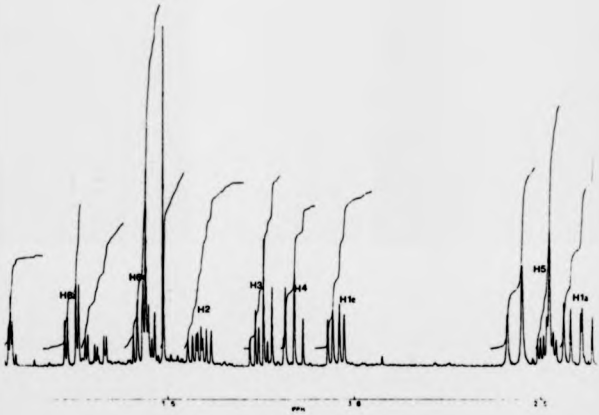


Figure 2.6. ^{1}H NMR spectrum of *S.lavendulae* 31434 product.

Proton	Splitting pattern	Shift (PPM)	Coupling constants (Hz)
H-1a	dd	2.4	12.34 (H-1e) 10.8 (H-2)
H-1e	dd	3.03	12.36 (H-1a) 5.16 (H-2)
H-2	ddd	3.4	5.16 (H-1e) 9.07 (H-3) 10.8 (H-1a)
H-3	t	3.24	9.07 (H-2) 9.06 (H-4)
H-4	t	3.15	9.7 (H-5) 9.18 (H-3)
H-5	ddd	2.48	2.97 (H-6a) 6.25 (H-6e) 9.58 (H-4)
H-6a	dd	3.77	2.98 (H-5) 11.67 (H-6e)
H-6e	dd	3.55	6.25 (H-5) 11.7 (H-6a)

Table 2.3. ^{1}H NMR assignments for *S.lavendulae* 31434 product.

protocols but were found to be impractical with the small amounts of material being handled in these experiments.

On the basis of the TLC of the final product it was possible to determine that the major component of the 31434 sample was DNJ. The assignments of the peaks in the ^{1}H-nmr spectrum (Fig. 2.6 and Table 2.3) were made with the aid of the COSY spectrum. The presence of DMJ is shown by the presence of the characteristic multiplet at 3.9ppm corresponding to the H-2 proton of DMJ. The H-2 proton of DNJ shows up as a ddd pattern at 3.4ppm. The large difference in the shift of these two protons is due to the change of stereochemistry at this position.

Both of these characteristic H-2 patterns are seen in the ^{1}H-nmr spectrum of the 445 sample (Fig. 2.7). The presence of DNJ is highlighted by the growth of its peaks in the spectrum upon addition of authentic sigma DNJ (Spectrum 445 + DNJ - Fig. 2.8). The positions of H-4 and H-3 of DNJ are reversed in this spectrum compared to the 31434 sample. The reason for this is unknown but may be due to the presence of a higher level of DMJ. In other spectra of the mixed isomers these peaks are overlapping and indistinguishable (D.Hardick personal communication). Both spectra were run under identical conditions and in the same solvent.

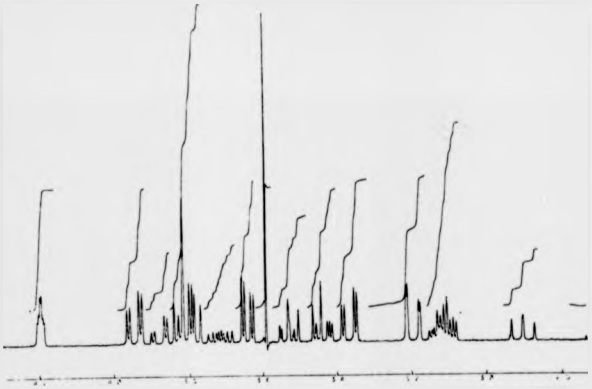


Figure 2.7. ^{1}H NMR spectrum of 445 product.

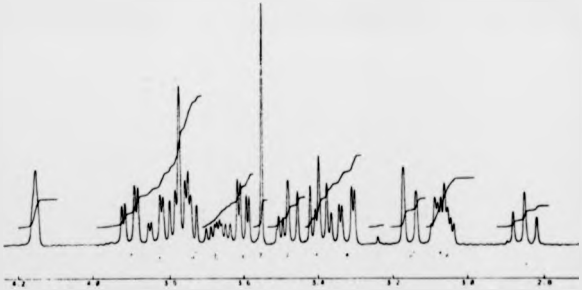


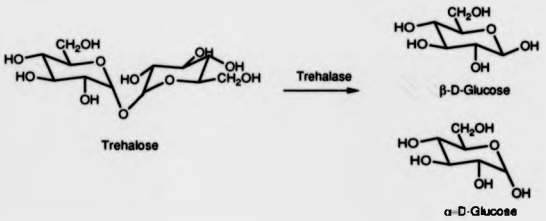
Figure 2.8. ^{1}H NMR spectrum of 445 product spiked with authentic DNJ.

It is interesting to note that both of the strains found to produce DNJ in this screen, *S.racemochromogenes* 194 and *S.subrutilus* 445, are taxonomically very closely related to the patented producer *S.lavendulae* ATCC 31434. Ezure conducted a screen where of 8500 *Streptomyces* Strains isolated freshly isolated from soil, all 96 strains found to produce DNJ were identified as *S.lavendulae* (Ezure *et al.*, 1985).

2.3.2. USE OF TREHALASE IN QUANTITATIVE ASSAY OF DNJ.

INHIBITION OF TREHALASE BY DNJ AND NJ.

The enzyme employed in the assay was Pig kidney α,α-trehalase (EC 3.2.1.28) obtained from Sigma. The protein has a molecular weight of 80 000 and an optimal pH of 5.9 (Yoneyama, 1987). Trehalases are highly specific for trehalose (glucopyranose-α,α-glucopyranoside) as substrate (Alabran *et al.*, 1983; Yoneyama, 1987) and catalyse the reaction shown in equation 1.



Equation 1. The trehalase reaction.

The high degree of specificity shown by trehalase is an advantage in such an assay as the concentration of substrate for the reaction is unlikely to be altered by the presence of trehalase in the fermentation samples in the same way that the potential substrate concentration for a less specific glucosidase might be. Other glucosides in the fermentation sample might act as substrates for the enzyme, thereby increasing the effective substrate concentration and the reaction rate in the sample run with the inhibitor. This would lead to the assay becoming inaccurate but is unlikely to occur with the trehalase assay despite trehalose being used as a storage material in the spores of some microorganisms (McBride and Ensign, 1987). However as both the trehalase and its substrate will remain intracellular they should not interfere with this assay which is concerned only with extracellular materials.

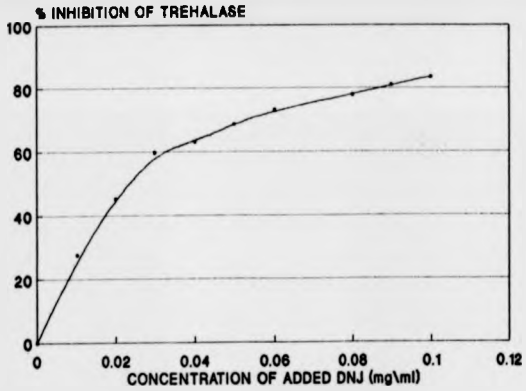


Figure 2.9. Inhibition of trehalase by DNJ.

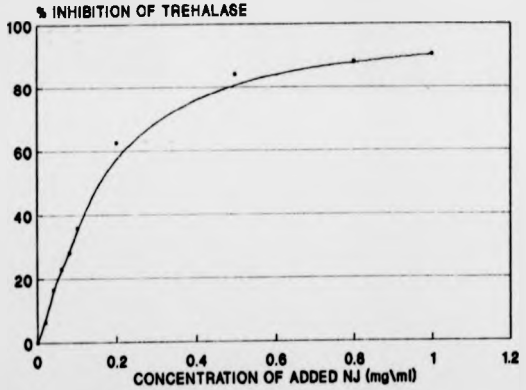


Figure 2.10. Inhibition of trehalase by NJ.

The results (Figs 2.9, 2.10) show that both DNJ and NJ are inhibitors of pig kidney trehalase (EC 3.2.1.28). DMJ was found to be inactive against the enzyme. This shows that the stereochemistry at the C-2 position of the inhibitor is an important factor in the recognition of the inhibitor by the enzyme. The inhibition kinetics (Figs. 2.11, 2.12) show the competitive nature of this inhibition at pH 6.0. This is not surprising since both resemble the glucose subunits of the trehalose substrate. DNJ is a stronger inhibitor of the pig kidney trehalase than NJ as is shown by the K_i and IC_{50} values (Fig. 2.13) which are both around 10-fold smaller for DNJ than NJ. DNJ is reported to inhibit a trehalase in mouse small intestinal mucosal homogenate with an IC_{50} value of 6.7×10^{-5}M (Schofield *et al.*, 1986) and a rabbit intestinal trehalase with an IC_{50} of 3.4×10^{-5}M (Nishimura *et al.*, 1990). Both of these values are comparable to the one obtained for DNJ on the pig kidney trehalase obtained in this study.

The Km value calculated from the inhibition kinetics is 1.7mM which is comparable to the literature value of 2.1mM (Yoneyama, 1987). The V_{max}, also calculated from the kinetic data, was 4×10^{-3} µmol\min.

Figure 2.11. Lineweaver-Burk plot of inhibition of trehalase by DNJ.

Figure 2.12. Lineweaver-Burk plot of inhibition of trehalase by NJ.

INHIBITOR	IC_{50} (M)	K_I (M)
DNJ	4×10^{-5}	3.45×10^{-6}
NJ	2.9×10^{-4}	2.6×10^{-5}

Km= 1.7mM

V_{max}= 4 x 10^{-3} µmol min^{-1}.

Table 2.4. Kinetic constants for the inhibition of trehalase by DNJ and NJ.

Little is known about the basis of the inhibition of trehalase by DNJ and NJ but some idea of how they might act can be gained from looking at the mechanism of action and their inhibition of other similar glucosidases. The mechanism of action of intestinal sucrase has been proposed (Cogoli and Semenza, 1975) and is shown below (Fig. 2.13). The competitive inhibition of sucrase by DNJ and NJ (Figs. 2.11, 2.12) probably arises from the resemblance of the protonated form of the inhibitor to the substrate in the enzyme-

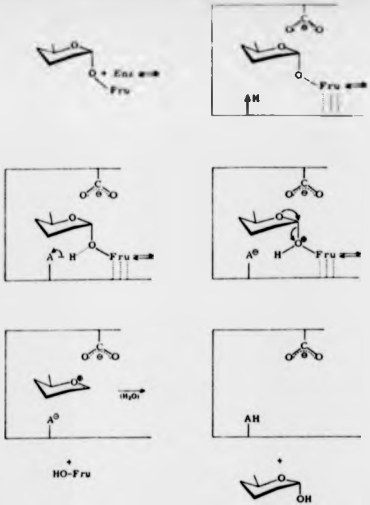


Figure 2.13. Mechanism of intestinal sucrase (Cogoli and Semenza, 1975)

substrate complex. The mechanism involves the formation of a charged species from the substrate which could be mimicked by the protonated inhibitor. The formation of an ion pair between the inhibitor and the carboxyl group at the active site would serve to strengthen its binding. Such electrostatic interactions have been implicated in the inhibition of calf glucosidase I by DNJ (Schweden *et al.*, 1986) and in the inhibition of α-fucosidase by DMJ (Winchester *et al.*, 1990). The protonation of the inhibitor

has been shown to be very important in the inhibition of β-glucosidase (Legler, 1978). Where analogues which were not able to become protonated were used, they showed little inhibition compared to active inhibitors. It was also demonstrated that when a species was already protonated it could not act as an inhibitor thereby indicating the presence of another positively charged group in the active site of the enzyme which would repel an already protonated inhibitor. A slow onset of inhibition of sucrase by DNJ and NJ has been noticed (Hanozet *et al.*, 1981) and proposed to be due to a two stage process of inhibition by these compounds. The first stage is proposed to be the initial binding of the inhibitor at the active site of the enzyme, and the second is the protonation of the inhibitor at the active site leading to a strengthening of the forces holding the inhibitor at its binding site. To account for this, a 15 minute pre-incubation of the trehalase with the inhibitor was included in the assay protocol.

The implication of carboxyl groups and an imidazole group in catalysis by Cockchafer trehalase (Defaye *et al.*, 1981) has led to the proposal of a mechanism for the action of the enzyme (Defaye *et al.*, 1983). The mechanism is shown in Figure 2.14.

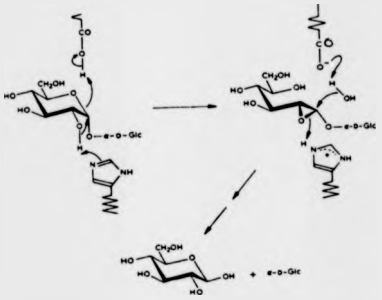


Figure 2.14. Trehalase mechanism proposed by Defaye *et al.* (1983).

Results from the study on the effect of pH on the inhibition of pig kidney trehalase (see later in this chapter) indicate that the protonated form of the inhibitor is the more active. This observation correlates well with the above mechanism. Defaye envisages the substrate binding to an active site containing a charged carboxyl group and an uncharged imidazole group. Protonation of the inhibitor would therefore mean that ion-pair formation between the enzyme and inhibitor

would occur and stabilise the complex. The uncharged imidazole group would not repel the incoming and already charged inhibitor in the way that is hypothesised for the active site of β-glucosidase (Legler *et al.*, 1978).

EFFECT OF ACID AND HEAT TREATMENT ON INHIBITION OF TREHALASE BY DNJ AND NJ.

The observation that NJ as well as DNJ was able to inhibit trehalase in the assay, albeit with an IC_{50} value ten fold larger than that for DNJ (Table 2.4) meant that assaying DNJ using the existing assay would give a false result when NJ was present in the fermentation. Two solutions were available, firstly to assay using a selective method such as the gas chromatographic (GC) method already described which does not detect NJ or secondly, to find a way to remove the effects of NJ from the sample. For reasons which will be discussed later the second approach was adopted.

In 1968 (Inouye *et al.*, 1968) it was reported that when heated with HCl, NJ underwent the reaction shown in Figure 2.15.

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Figure 2.15. Effect of acid on nojirimycin molecule.

Results of the experiments (Figs. 2.16, 2.17) show that the ability of NJ to inhibit trehalase declines sharply upon heating with HCl at 70 or 90°C. The ability of DNJ to inhibit the enzyme is, however, virtually unaffected by the same treatment. Weaker concentrations of NJ were more quickly destroyed by the treatment and 6M HCl at 90°C was generally found to be more efficient than 3M at 70°C. On the basis of these results a pre-treatment was adopted to remove the problem of interference by NJ when assaying DNJ by the trehalase method. The procedure involved the heating of the sample with an equal volume of 6M HCL at 90°C for 6h . The sample was then freeze-dried to remove the HCl and resuspended in distilled water. The heat treatment could not be guaranteed to remove totally the effects of NJ concentrations over 1mg\ml but such concentrations were not

anticipated at this stage. Apart from removing interference by NJ the heat and acid treatment had the added advantage of

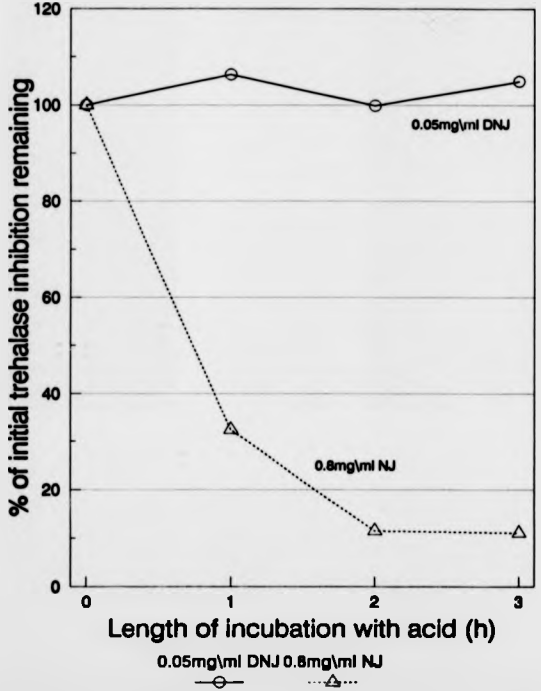


Figure 2.16. Inhibition of trehalase by DNJ and NJ: Effect of incubation with an equal volume of 3M HCl at 70°C

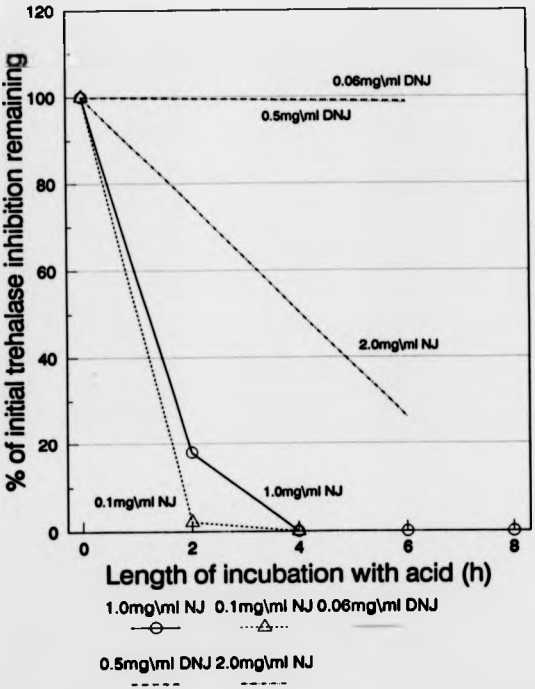


Figure 2.17. Inhibition of trehalase by DNJ and NJ: Effect of incubation with an equal volume of 6M HCl at 90°C.

denaturing any enzymes such as proteases or maybe even trehalase which would also interfere with the assay. The inclusion of the heat\acid treatment should allow the use of the trehalase reaction to quantify DNJ in fermentations.

EFFECTS OF pH ON THE TREHALASE ASSAY.

Due to the effect of the protonation of inhibitors such as DNJ on their inhibitory properties, the pH of the incubation medium also has a marked effect. This has been shown for the inhibition of β-glucosidase (Dale *et al.*, 1985) and intestinal sucrase (Hanozet *et al.*, 1981) by DNJ and also for the inhibition of various enzymes by castanospermine (Saul *et al.*, 1984). In all these cases, pH values below the pKa for the inhibitor showed a lower degree of binding and inhibition than those above indicating that it is the unprotonated form of the inhibitor which is active.

This is not the case for all enzymes though. The effect of pH on the inhibition of calf liver glucosidase I indicates that it is the protonated form of DNJ which is the potent form against this enzyme (Schweden *et al.*, 1986). The protonated form of DMJ is also believed to be the active form in the inhibition of α-fucosidase by this inhibitor (Winchester *et al.*, 1990).

The variability of the effect of pH on inhibition by DNJ of different enzymes makes it important to assess which pH gives the highest degree of inhibition of trehalase in order to achieve maximum sensitivity of the assay. The results of this study (Fig. 2.18) show that pH 6.0 gave the highest inhibition and so should be used for the assay.

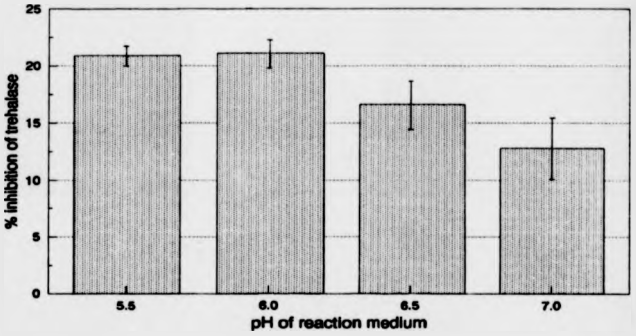


Figure 2.18. Effect of pH on inhibition of trehalase by DNJ.

The highest amounts of inhibition were seen at pH 5.5 (20.9%) and 6.0 (21.1%), at pH 6.5, this fell to 16.6% and at pH 7.0 is was lower still at only 12.8%. As the pKa for DNJ is 6.4 (Schweden *et al.*,1986), these results would seem to indicate that it is the protonated form of DNJ which is inhibiting the pig kidney trehalase, although determination of K_i values should be calculated for each pH value before definite conclusions are drawn.

EFFECTS OF BACKGROUND GLUCOSE CONCENTRATION ON THE TREHALASE ASSAY.

As glucose is a product of the trehalase reaction then high levels of this compound in the fermentation sample being assayed might be expected to cause a disturbance such as feedback inhibition to the enzyme. No such effect was seen in these studies (Fig. 2.19). With the concentration of glucose in the added solutions of up to 0.6 mg\ml, the only effect was a slight activation of up to 2% of the activity of the enzyme. At concentrations of between 0.6 and 1.0mg\ml the activation rose to between 5.96 and 9.6% and at 2.0 mg\ml it was 34.2%. The apparent activation of the trehalase in the presence of the higher concentrations of glucose would mask some of the inhibition caused by any DNJ present. For this reason, samples containing glucose at a concentration likely

to cause such an interference were diluted to a level where the problem would be avoided prior to assay.

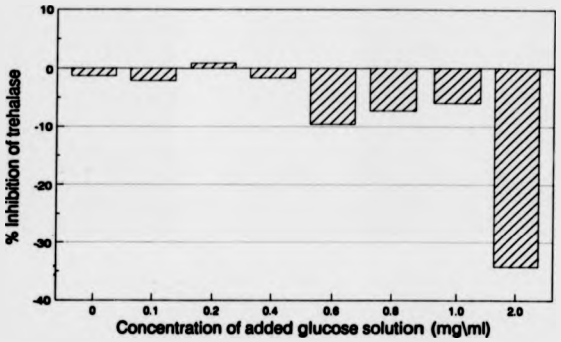


Figure 2.19. Effect of glucose concentration in sample on trehalase assay.

2.3.3. COMPARISON OF THE TREHALASE ASSAY OF DNJ WITH THAT BY GAS CHROMATOGRAPHY.

The trehalase and the GC methods of DNJ assay were compared by assaying three different solutions using the two methods and then comparing the results (Fig. 2.20).

SAMPLE ASSAYED	DNJ BY GC (µg\ml)	DNJ BY TREHALASE (µg\ml)
0.05mg\ml DNJ in distilled water		
After heat\acid treatment	53.4 ± 8.41	56.3 ±13.67
TK24 fermentation spiked with 0.05mg\ml DNJ		
Before heat\acid treatment	45.1 ± 2.51	45.6 ± 6.7
After heat\acid treatment	62.0 ±2.36	44.3± 8.5
Strain 446 fermentation		
Before heat\acid treatment	63.5 ± 4.95	90 ±11.13
After heat\acid treatment	68.0 ± 8.13	61.6 ± 9.29

Figure 2.20. Comparison of assay results fom trehalase and GC assays.

The figures for the concentration of DNJ in a 0.05mg\ml solution of DNJ in distilled water are comparable to each other and close to the correct concentration. This shows that the both assays give similar and accurate values for DNJ. The two assays also give comparable figures when assaying a 0.05mg\ml DNJ solution made up in TK24 fermentation supernatant. The figures reached were a little lower than the expected value but may just reflect inaccuracies in the measurements when making up the solution. The concentration by trehalase assay was unchanged after heat treatment showing that heat treatment in the fermentation medium, as in distilled water, had no effect on the inhibition of trehalase by DNJ. The reason for the rise in the amount of DNJ assayed by the GC method after heat treatment is unknown. The GC assay of the 445 fermentation, as expected gave very similar figures both before and after heat treatment, as NJ present does not interfere with this assay. The figure given by the trehalase assay before heat treatment was a lot higher than this, the overestimate being due to the NJ present. After heat treatment, however, the figure was almost the same as that for the GC assay, as the interfering NJ had been removed.

Although both assays were found to be equally as accurate for the measurement of DNJ concentrations they were not equally as applicable to all samples to be assayed. The GC method has the advantage of not requiring the pre-treatment of samples but when the samples to be assayed are from fermentations in

defined medium without purification, the time taken to elute all the residue from one sample before the next is injected severely limits the throughput of the system. An additional problem associated with the assay of complex medium samples is the tendency, when many samples are being injected, for the splitter on the injector to become blocked with involatile residue, causing overloading of the column. The use of a purification step prior to GC assay may help this situation but runs the risk of introducing inaccuracy through loss of DNJ. For these reasons, the trehalase assay, with a larger throughput and none of the problems associated with complex media and the GC method was used for assays involving such media.

When the samples were from fermentations in defined media, however, the GC assay method was not as disadvantageous. The throughput was increased to 10 minutes between sample injections compared to over 20 minutes with complex media and the lack of the need for the acid\heat treatment avoided the problem of high acidity sometimes seen with the defined media samples after such treatment and which caused problems with the trehalase assay.

So, in general, the trehalase was used for assays of samples in complex media, especially when there were many to be assayed and the GC assay was used for samples in defined media.

CHAPTER 3. INFLUENCE OF NUTRIENTS AND CULTURE CONDITIONS ON THE PRODUCTION OF DNJ.

3.1. INTRODUCTION.

The nature and quantity of secondary metabolites produced by an organism during a fermentation is influenced by a great number of factors (Zahner and Kurth, 1982; Vining, 1986; James and Edwards, 1988; Vilches *et al.*, 1990; Hobbs *et al.*, 1990; Liefke *et al.*, 1990). In rich media allowing maximal growth of the organism, the production of secondary metabolites is usually limited to late stages in the fermentation, when the period of fast growth is over. The depletion of one or more of the essential nutrients in the medium may therefore be required for the initiation of secondary metabolism. The terms "trophophase" and "idiophase" have been coined to describe the periods of fast growth and secondary metabolite production respectively (Bu'Lock, *et al.*, 1961). In media where only slow growth is supported, for example due to the supply of an essential nutrient in a limited or only slowly utilisable form, then secondary metabolite formation during the growth of the organism can be observed (Haavik, 1974a; Aharonowitz and Demain, 1979; Shapiro and Vining, 1984).

When studying a particular metabolite, the generation of sufficient quantities of the compound is very important. The improvement of the product titre can be approached in several ways. The genetics of the organism can be altered, usually by random mutation followed by selection of overproducing mutants, or the conditions for the fermentation can be optimised. The second approach is probably best to start off with since it involves less of a chance element and is likely to yield an improvement in titre in a shorter space of time. The genetic would probably yield greater improvements in production in the long run but be more time consuming, especially if little or nothing of the biosynthetic origins or regulation of production of the metabolite is known. This approach is probably better left for situations where the metabolite has already been studied and its potential value assessed. An improvement in fermentation conditions via the identification of key nutrients are able to either stimulate or suppress the production of the metabolite could be carried out fairly early on in the investigation of a compound and the knowledge obtained may still be useful if the strain improvement process is carried out later.

CARBON CATABOLITE SUPPRESSION OF SECONDARY METABOLITE PRODUCTION.

The nature of the major carbon source in a medium can have a dramatic effect on the amount of secondary metabolite production in that medium (Demain, 1982; Demain, 1989a). Generally carbon sources which are readily assimilated and can support rapid growth of an organism suppress both the utilisation of other carbon sources and the production of secondary metabolites, although the mechanisms for these two effects are probably different (Vining, 1986). Glucose is a very good carbon source for the growth of many organisms, for example, but suppresses the production of a large number of secondary metabolites including erythromycin (Escalante *et al.*, 1982), oleandomycin (Vilches *et al.*, 1990) and actinomycin (Gallo and Katz, 1972). In a medium where two carbon sources are provided, one of which is glucose, the glucose is generally used first accompanied by a period of rapid growth. When the glucose is depleted, the utilisation of the second carbon source begins and secondary metabolite production may commence. If a carbon source other than glucose is favoured for growth then that may suppress the secondary metabolite production. This is the case in *S.clavuligerus*, where glycerol is able to suppress the production of candicidin (Hu *et al.*, 1984). The suppressive action of a particular carbon source may be overcome by the use of a more slowly utilised source such as starch in the

case of the glucose suppression of cephamycin production (Aharonowitz and Demain, 1978) or by a slow feeding regime of the suppressive source so that it does not reach suppressive levels. The second approach has been applied to the production of penicillin (Soltero *et al.*, 1954).

The usual mode of action of carbon catabolite suppression is repression of the biosynthetic genes for the metabolite (Demain, 1989a), which can take place at the level of transcription, translational, or in the case of eukaryotic fungi, at the level of message splicing or post-translational modification of the protein. Repression can be shown to be the mechanism when the suppressive action is only exerted at stages in the fermentation prior to the expression of the biosynthetic genes. In some cases, the repression of individual enzymes has been demonstrated, this is so with phenoxazinone synthetase in actinomycin production by *S.antibioticus* (Jones, 1985), the expandase of cephamycin production (Lebrihi *et al.*, 1988a) and the O-demethyl puromycin O-methyl transferase of puromycin biosynthesis (Sankaran and Pogell, 1975), for example. Enzyme inhibition by the carbon source may also contribute to the suppression of the secondary metabolite production. Carbon regulation of cephalosporin and cephamycin production has been reported to be due to both repression and inhibition of the enzyme deacetoxycephalosporin C synthetase involved in their biosynthesis (Martin *et al.*, 1986).

NITROGEN SOURCE REGULATION OF SECONDARY METABOLITE FORMATION.

As with the carbon source, the form in which nitrogen is supplied to a culture also influences the production of secondary metabolites (Aharonowitz, 1980; Shapiro, 1989). Ammonium and other inorganic nitrogen sources which are easily assimilated and support fast growth tend to suppress production of antibiotics and other secondary metabolites (eg. Flores and Sanchez, 1985; Demain and Piret, 1991; Held and Kutzner, 1990; Hobbs *et al.*, 1990; Doull and Vining, 1990; Truck *et al.*,1990a). Nitrogen sources which are assimilated slowly allow a much slower growth rate and the production of secondary metabolites. For example, the production of novobiocin by *S.niveus* is suppressed by ammonium so that novobiocin is not produced until the ammonium in the medium is depleted. Substitution of the ammonium with proline results in a considerable increase in novobiocin titre (Kominek, 1972). The addition of ammonium to a medium in a poorly accessible form is also a way to avoid its suppressive effects If ammonium is supplied to a fermentation the tylosin producer, *S.fradiae* as the sparingly soluble ammonium magnesium phosphate instead of the readily soluble ammonium lactate, then an increase in the production of tylosin is seen (Omura *et al.*, 1980). Another way to

overcome ammonium suppression is to add an ammonium complexing agent such as zeolite to the medium which then allows only its slow release and assimilation (Omura and Tanaka, 1986). The effect of ammonium on the production of secondary metabolites is always by repression, unlike carbon regulation, enzyme inhibition is not involved (Flores, 1991).

PHOSPHATE REGULATION OF SECONDARY METABOLITE PRODUCTION.

Suppression of secondary metabolite biosynthesis by excess phosphate is another common phenomenom in the pathways which have been studied so far (eg. Liras *et al.*, 1977; Mertz and Doolin, 1973; Shapiro and Vining, 1983; Zhang *et al.*, 1989, Hobbs *et al.*, 1990; Kirpekar *et al.*, 1991). Again, the depletion of inorganic phosphate is required before the onset of secondary metabolism. Production of certain groups of secondary metabolites is more sensitive to phosphate repression than others, amongst the more sensitive pathways are those resulting in the formation of the aminoglycosides, the macrolides and the tetracyclines (Martin, 1989).
During the fermentation of *S.griseus*, phosphate is almost depleted from the medium before any candicidin is made, and further additions of phosphate after the onset will stop production (Liras *et al.*, 1977; Martin and Demain, 1976). β-

Lactam biosynthesis by *S.clavuligerus* is another example of a system which is affected by the phosphate concentration. The four main biosynthetic enzymes are all repressed by high concentrations of phosphate, which also acts as an inhibitor of the expandase and cyclase enzymes (Zhang *et al.*, 1989). The amount of phosphate can also affect the relative amounts of secondary metabolites made, for example as its suppressive effect on clavulanic acid production is greater than on cephamycin (Romero *et al.*, 1984) and in *S.coelicolor* A3(2), 24mM phosphate will completely prevent the biosynthesis of actinorhodin, without affecting that of undecylprodigiosin (Hobbs *et al.*, 1990). The effect of phosphate on the synthesis of gramicidins also involves the inhibition of enzymes involved in their biosynthesis since the addition of phosphate to a fermentation after the synthesis of gramicidin S has already started results in no further synthesis and a drop in the activity of the enzymes until the level of phosphate has dropped again (Behal, 1986).

In the streptomycin producer, *S.griseus*, excess phosphate encourages the accumulation of a phosphorylated precursor to streptomycin (Miller and Walker, 1970) due to the inhibition of the final enzyme in its biosynthesis which cleaves the phosphate group from the precursor to yield streptomycin (Walker and Walker, 1971). The biosynthesis of other aminoglycoside antibiotics, such as neomycin and viomycin, also involves the use of phosphorylated intermediates. These antibiotics are also subject to phosphate regulation (Martin,

1989) and a similar effect to that with streptomycin is seen with the inhibition of an alkaline phosphatase, which is involved in the biosynthesis of neomycin B in *S.fradiae*, by phosphate (Demain, 1982).

Apart from repression and inhibition of enzymes involved in the biosynthesis of aminoglycosides, where phosphate is involved as a substrate or product of the reaction, enzymes for other pathways where phosphate acts in neither of these capacities are also phosphate repressed. Amongst these are p-aminobenzoic acid synthetase acting in the biosynthesis of candicidin in *S.griseus* (Gil *et al.*, 1985) and several enzymes involved in the biosynthesis of both the macrolide and the aminosugar parts of the antibiotic tylosin in *S.fradiae* (Madry and Pape, 1982).

Severe suppressive effects of phosphate concentration on secondary metabolite production can be removed by the utilisation of only a limited concentration of phosphate in the medium or the addition of a phosphate complexing reagent such as Kanuma earth (Omura and Tanaka, 1986) which would allow only the slow release of phosphate into the medium thereby avoiding high concentrations of inorganic phosphate.

THE RELATIONSHIP BETWEEN GROWTH RATE AND THE PRODUCTION OF SECONDARY METABOLITES.

The observation that the production of secondary metabolites is usually limited to fermentation conditions which permit only suboptimal growth rates begs the question of whether this factor alone can act to regulate secondary metabolism. Although the growth rate of an organism may well depend on the concentration of a repressive nutritional element, such as phosphate, in the medium, and therefore is linked to it, there is evidence that the growth rate *per se* might have a regulatory role in some systems (Vining, 1986). The commencement of secondary metabolism in response to a non-specific nutrient limitation, as is often seen with the peptide group of antibiotics, might be seen as an indication that growth rate is playing a regulatory role. The importance of growth rate as a regulatory factor is dependant on the metabolite concerned. *S.cattleya*, for example, produces both cephamycin C and thienamycin but whereas the production of both these metabolites requires a low growth rate, this must also be accompanied by phosphate limitation for thienamycin production. The low growth rate needed for cephamycin C production can be due to depletion of carbon or nitrogen source or inorganic phosphate from the medium (Lilley *et al.*, 1981). Thus for thienomycin production the low growth rate is probably a result of the depletion of phosphate which is the stimulus for the onset of the thienomycin production. Low

growth rate alone appears to be the stimulus for cephamycin C production. This is corroborated by the observation that there is an inverse relationship between growth rate and cephamycin C production irrespective of the carbon source employed (Lebrihi *et al.*, 1988b).

EFFECT OF TRACE ELEMENTS ON SECONDARY METABOLITE BIOSYNTHESIS.

The trace element content of a medium can have important effects on the production of secondary metabolites, although some trace elements, such as zinc, manganese and iron are more important than others (Weinberg, 1982; Weinberg, 1989). The trace element composition can have a stimulatory or an inhibitory effect on the biosynthesis of a particular secondary metabolite depending on the the concentration. The effect may be due to a single element or a combination of two or more.

As with the other nutrient conditions discussed, the effect on the production of secondary metabolites by various trace metals is very much dependant on the metabolite and the organism producing it. This is exemplified by the production of peptide antibiotics by three species of *Streptomyces*, *S.albus*, *S.albulus*, and *S.griseoincarnatus* (Miyashiro and Udaka, 1983). Addition of zinc to cultures of these organisms increased the production of antibiotic by *S.albus* and

S.albulus, but decreased it in the other strain whereas magnesium additions were found to have the opposite effect. The addition of manganese stimulated production in *S.albulus* and *S.griseoincarnatus* but not *S.albus* and high levels of calcium increased production by all three. The addition of cobalt to a fermentation of *Micromonospora sagamiensis* alters the ratio of three gentamycins made by this organism without altering their overall yield (Kase *et al.*, 1982).

The effect of the different trace elements may be due to their role as co-factors or allosteric effectors for enzymes of either primary or secondary metabolism. In *S.michiganensis*, copper ions are needed for the activity of tyrosinase, the enzyme responsible for the production of the secondary metabolic pigment melanin. In addition to this, they are also found to induce the expression of this enzyme (Held and Kutzner, 1990). In *S.coelicolor*, growth and actinorhodin production is affected by the trace element status of the medium (Abbas and Edwards, 1990). Elements such as lead, copper and nickel inhibit both growth and antibiotic production but zinc, manganese, cobalt and chromium enhance growth and inhibit actinorhodin production. Calcium will inhibit actinorhodin production and enhance growth but only if added early in the fermentation, indicating that its effect on antibiotic production is mediated through repression rather than inhibition of the biosynthetic enzymes.

TEMPERATURE AND pH EFFECTS.

As changes in both temperature and pH of a medium affect both the growth rates of organisms and the activity and half life of their enzymes, they might also be expected to have an effect on the production of secondary metabolites.

The pH of the medium was claimed to be the effector in the interference by glucose in the biosynthesis of bacitracin by *Bacillus licheniformis*. The organic acids which built up on the fast metabolism of glucose were believed to cause a lowering of the pH and a decrease in the synthesis of bacitracin (Haavik, 1974b). This effect could be mediated via the alteration of the conformation of an activator protein so that it cannot switch on the expression of the bacitracin biosynthetic genes under these conditions. The drop in pH may also alter the activity of any biosynthetic enzymes already synthesised. The pH optima for many of the streptomycin biosynthetic enzymes has been found to be the same as the pH optimum for streptomycin biosynthesis. Changes in the pH during the fermentation of *S.thermoviolaceus* were found to affect both the timing and amount of granaticin produced. A rise in pH was found to correlate with the onset of production (James and Edwards, 1988).

Temperatures giving optimal growth rates have also been found to limit the production of secondary metabolites, with the optimal temperature for the synthesis of the biosynthetic

enzymes being different to that for their optimum activity, generally, the best temperatures for secondary metabolite formation are lower than those for optimum growth (Weinberg, 1982; Weinberg, 1989). This indicates that the effect of different temperatures may be related to their effect on the growth rate of the organism and expression of the antibiotic biosynthetic genes rather than the activity of the enzymes. Streptomycin production by *S.griseus* is much deminished at 34-37°C due to alterations in the transcription of the biosynthetic genes (Baumberg *et al.*, 1991).

EFFECT OF OXYGEN TENSION ON SECONDARY METABOLITE PRODUCTION.

Reduced oxygen tension has been associated with effects such as a drop in the specific activity of the enzyme deacetoxycephalosporin C synthetase in *S.clavuligerus* (Rollins *et al.*, 1988) and a lower yield of aristeromycin in *S.citricolor* (J. Hanrahan, pers. commun.). Production of tetracyclines by *S.aureofaciens* and *S.rimosus* has also been shown to increase at higher oxygen tension (Liefke *et al.*, 1990) and limited diffusion of oxygen has been blamed for the drop in production of nikkomycins by *S.tendae* immobilised on glass beads when the thickness of the layer of organisms on

the beads is increased (Truck *et al.*, 1990b). Generally a higher oxygen tension appears to be beneficial for secondary metabolite production, although oxygen limitation has been linked to induction of erythromycin biosynthesis by *Saccharopolyspora erythraea* (Clark *et al.*, 1991).

Studies on the effect of oxygen tension on the production of antibiotics by *S.clavuligerus* in defined media have shown that the drop off of antibiotic titre after its peak level is increased in reduced oxygen conditions. This was hypothesised to be due to derepression of antibiotic hydrolases under the low oxygen conditions (Yegneswaran *et al.*, 1988). A similar effect may be responsible for the link between lower oxygen tensions and lower antibiotic titres described above.

EFFECTS OF SPECIFIC NUTRIENTS ON SECONDARY METABOLITE PRODUCTION - PRECURSOR EFFECTS.

Apart from the general effects of carbon, nitrogen and phosphate suppression of secondary metabolism, certain primary metabolites, usually amino acids, have been found to specifically increase or decrease the production of a secondary metabolite (eg. Majumder and Kutzner, 1962; Yamamoto *et al.*, 1977; Mendelowitz and Aharonowitz, 1982; Williamson *et al.*, 1985; Goo *et al.*, 1991). One of the best examples is the stimulation of alkaloid production in

Claviceps by the amino acid tryptophan (Krupinski *et al.*, 1976). Tryptophan is a common precursor for (Kobel and Sanglier, 1986), and is also able to negate the suppressive effect of excess phosphate on alkaloid biosynthesis. Other examples include the stimulation of penicillin biosynthesis by glutamine a possible amino donor in its biosynthesis (Aharonowitz and Friedrich, 1980) and the induction of cephalosporin production in *Acremonium chrysogenum* by methionine (Drew and Demain, 1975).

The pathways of primary metabolism in the producing organism also play a role in the inhibitory of inductive effects of certain nutrients. Lysine, for example, acts as a stimulant for β-lactam production in *Streptomyces* but as an inhibitor of their production in fungi. The reason for this difference is the different routes for the biosynthesis of lysine in the two types of organism. In fungi, lysine is synthesised via α-aminoadipic acid (α-AAA), which is used in the biosynthesis of β-lactam antibiotics. When extra lysine is added, feedback inhibition of the pathway results in the cessation of the synthesis of α-AAA and also therefore β-lactams. In *Streptomyces*, the biosynthetic pathway to lysine does not involve α-AAA which is formed instead by the action of L-lysine amino transferase, a secondary metabolism enzyme (Madduri *et al.*, 1991) not involved in the usual pathway of lysine catabolism which proceeds via cadaverine (Madduri *et al.*, 1989).

End product inhibition of the shikimate pathway by aromatic amino acids is also responsible for the negative effect that these compounds have on the biosynthesis of candicidin (Gil *et al.*, 1985), an antibiotic which requires intermediates from this primary metabolic pathway for its biosynthesis.

AUTOREGULATORS.

The correlation between the developmental stages of antibiotic producing Actinomycetes and their production of secondary metabolites has led to the hypothesis that at least some of the secondary metabolites might act as effectors to control the differentiation events in the life cycle. This function may or may not have any relevance to the bioactivity associated with some of these compounds. Evidence that some of these compounds can indeed act as autoregulators has been found (Khoklov, 1982; Graffe, 1989). Although these compounds are not essential for the cell, they can have a profound effect on differentiation and secondary metabolite formation. The best known of these is A-factor from *S.griseus*, which was originally isolated as a factor capable of restoring the ability to sporulate and produce streptomycin to *S.griseus* mutants deficient in both of these functions. A-Factor is a γ-butyrolactone (Fig. 3.1) and has been detected in 15% of

examined Actinomycetes (Vining, 1990) although it might not have the same effect in all of these strains (Graffe, 1989). Other butyrolactones have also been found to affect antibiotic production and sporulation in *S.coelicolor* and *S.virginiae* indicating that the regulatory function might be a general property of this group of secondary metabolites (Graffe, 1989). Regulatory function is not, however limited to the butyrolactones, with both protein Factor C (Biro *et al.*, 1980), nucleoside Factor B (Kawaguchi *et al.*,1984), and the ionophoric pamamycin (McCann and Pogell, 1979) also being able to affect morphological changes in different actinomycetes.

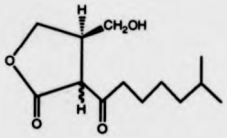


Figure 3.1. A-Factor.

MECHANISMS OF REGULATION.

It is unlikely that glucose, phosphate or ammonium moieties interact directly with genes to affect their expression and

so secondary messengers which might have such a role have been sought.

In some bacteria, a mechanism involving cyclic adenosine monophosphate (cAMP) is used to control the expression of primary pathways for the utilisation of different carbon sources. The binding of cAMP to a protein and then the binding of that complex to to a promoter of an operon for the utilisation of some carbon sources is inhibited by low cAMP concentrations. In the presence of glucose, the enzyme responsible for cAMP production, adenylate cyclase, is inhibited so that cAMP levels are kept low. Regulation by cAMP has been implicated in the production of certain secondary metabolites but the evidence is somewhat contradictory. For example, the cAMP level and tylosin production are both increased by the addition of an adenylate cyclase stimulator to the producer *S.fradiae* and a high producing strain was also found to have an elevated cAMP content (Colombo *et al.*, 1982). The onset of actinomycin production by *S.antibioticus*, however, is not accompanied by any rise in the cAMP level (Vining, 1986) and neither is the derepression of chloramphenicol production in *S.venezualae* (Chatterjee and Vining, 1982). Although the onset of streptomycin production in *S.griseus* is accompanied by a change, it is a fall and the cAMP concentration then remains low throughout idiophase (Ragan and Vining, 1978). Addition of cAMP to a fermentation was found to stimulate growth and abolish secondary metabolite production (Gersch, 1980) and

the effect of cAMP or adenylate cyclase activators in reversing the repressive effect of phosphate may be due to a stimulation of growth and consequent utilisation of excess phosphate.

Other nucleotide phosphates such as ppGpp, pppGpp and ATP have been looked at as possible secondary messengers. The levels of ppGpp and antibiotic production have been shown to be tightly coupled in mutants of *S.griseus* which are defective in their production of both aerial mycelium and antibiotics. They were found to have a lower concentration of ppGpp, which could be raised upon glucose starvation, which also stimulated aerial mycelium formation and antibiotic biosynthesis (Ochi, 1986). Similarly, in corresponding mutants of *S.coelicolor* A3(2), the level of ppGpp was 10-fold lower then in the wild-type (Ochi, 1990). As for ATP, the decrease in its intracellular concentration has been observed before the onset of antibiotic biosynthesis in *S.griseus* and the addition of phosphate to the fermentation will cause both the cessation of antibiotic synthesis and a concurrent increase in the concentration of ATP. A similar correlation between low ATP concentration and antibiotic biosynthesis has also been seen in other phosphate repressed systems. The observation of lowered ATP levels in overproducing mutants also points to an important role for this compound in control of antibiotic biosynthesis (Martin and Demain, 1980).

Apart from individual phosphorylated nucleosides, the control of secondary metabolism has also been linked to the total

adenylate pool, which takes into account the concentrations of ATP, ADP and AMP. It was found that chlortetracyline production by *S.aureofaciens* and tylosin production by *S.fradiae* both occurred after a fall in the total adenylate pool. Low levels of chlortetracycline production were associated with a high adenylate pool. Phosphate and glucose added to the medium both increased the adenylate pool (Vu-Trong *et al.*, 1980; Curdova *et al.*, 1976).

The enzyme glucokinase and its product glucose-6-phosphate are also likely candidates for regulatory effectors. Glucokinase mutants are less sensitive to glucose repression, a situation which can be reversed by the cloning of an intact glucokinase gene into the mutant (Seno and Chater, 1983; Ikeda *et al.*, 1984).

The proposed mechanisms of nitrogen catabolite repression have involved the enzymes of nitrogen assimilation as effectors (Aharonowitz, 1980). Under conditions of high ammonia, the nitrogen is assimilated using the enzyme glutamate dehydrogenase to make L-glutamic acid, but when, the ammonium level falls, it is assimilated using the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. In some organisms, alanine dehydrogenase has been demonstrated to have a nitrogen assimilatory role when ammonium levels are high (Aharonowitz and Friedrich, 1980). As the levels of all of these enzymes is controlled by the ammonium concentration, it may be that one or more of them plays a part in ammonium regulation of secondary metabolism.

However, mutants deficient in glutamine synthetase, glutamate synthase or alanine dehydrogenase are still subject to ammonium repression so these enzymes are unlikely to be shown to have a role in this phenomenom (Demain and Brana, 1986).

SUMMARY.

The nutrient status of a medium significantly affects the production of secondary metabolites. In general, media supporting only suboptimal growth rates will allow the production of secondary metabolites although the precise requirements vary considerably according to the metabolite and the species concerned. When studying a secondary metabolite, identification of suppressive or stimulatory elements is important so that the design of a medium giving sufficiently high levels of the metabolite can be achieved. The different sensitivities of the metabolites to the same limiting factor may also lead to alterations in the type and relative concentrations of metabolites made under different concentrations of that factor by a single strain. The precise mechanism involved in suppressive effects of nutrients such as ammonium, glucose and phosphate is unknown but it seems likely that ATP and/or other adenylates and ppGpp play an important role in this.

The aim of this study was to identify nutrient factors affecting the production of DNJ. In addition to this a medium was needed for studies on the biosynthetic pathway using isotopically labelled glucose. For these studies, a medium

was needed using glucose as the sole carbon source and producing sufficient DNJ for isolation and analysis in the smallest volume of medium and with the biggest yield from the added glucose.

3.2. MATERIALS AND METHODS.

3.2.1. ASSAY PROCEDURES.

DETERMINATION OF DNJ BY TREHALASE ASSAY.

Standard assay conditions were used to assay samples both before and after heat\acid treatment (See section 2.2.3). The values for percentage inhibition of trehalase were then converted into concentration of DNJ using the standard curve of inhibition of trehalase by DNJ solutions of different concentrations (Fig. 2.9). Samples containing over 1mg\ml glucose were diluted with distilled water prior to assay to a concentration where the glucose would have no effect on the assay. Samples giving an inhibition of trehalase of over 60% were diluted so as to give a range of values from the graph. This was done as at inhibitions over 60% a smaller inaccuracy in the % inhibition would give a much larger inaccuracy in the concentration of DNJ read from the graph than at other points. All assays were carried out in at least triplicate.

DNJ DETERMINATION BY GAS CHROMATOGRAPHY.

Determination of DNJ by GC was carried out as in section 2.2.4 except that the concentration of the methyl-ß-D-glucoside solution was varied so that the amount of standard added was roughly equal to the anticipated concentration of DNJ. The concentration usually used for the defined medium samples was 10 µg\ml.

GLUCOSE DETERMINATION.

Glucose was determined using the Peridochrom GOD-PAP glucose assay kit from Boehringer. The kit solution (1000µl) was added to the sample solution (20µl) and incubated at 37°C for 30 minutes. The absorbance of the red solution produced was read at 510nm in a Pye-Unicam 1800 UV Spectrophotometer. Each sample was assayed in triplicate and the absorbances converted to glucose concentration by means of a standard curve, constructed using known concentrations of glucose under the above conditions.

DETERMINATION OF NJ.

As NJ is an antibiotic, it was possible to assay it using an agar diffusion type bioassay. First, however, it was necessary to check that NJ was the only substance produced by the strains which can inhibit the growth of the sensitive strain, *Micrococus luteus* PCI1001. This was carried out with the use of bioautography.

Fermentations (30ml) of strains *Streptomyces lavendulae* 31434 and *S.nojiriensis*, which are known to produce NJ, *S.subrutilus* 445 which might, and *S.lividans* TK24 which does not, were grown in soyabean medium (Section 2.2.2) at 28°C for 6 days. They were then centrifuged (3000 rpm for 10 minutes in a Beckman JA20 rotor) and the supernatant used for the bioautography. The samples (10 x 10µl) along with standard solutions of nojirimycin (Gift of D.J. Hardick) were applied to a silica T.L.C. plate which was then developed with an ethanol:water (4:1) solvent system. After development, the plate was dried and laid face down on a 23cm square petri dish containing nutrient agar (150ml). After 1 hour, the silica plate was removed and the agar overlayed with 70% nutrient agar (50ml) seeded with *Micrococcus luteus* PCI1001 at a concentration of 10^6 cfu\ml. The plate was incubated at 30°C overnight and then inspected.

Having established that NJ was the only *M.luteus* growth inhibiting compound produced by *S.lavendulae* 31434, *S.nojiriensis* and *S.subrutilus* 445, the bioassay of NJ was

performed as follows. A 150ml layer of nutrient agar in a 23cm square dish was overlayed with 50ml 70% nutrient agar containing *M.luteus* at 10^6 cfu\ml. Sterile 1cm lengths of silicone tubing were then inserted into the top layer of the agar and the samples (50µl) added, both neat and 1/5 dilutions, along with standard NJ solutions in a quasi latin square arrangement. The plate was incubated at 30°C overnight and then the diameters of the clear zones around the tubes were measured. NJ concentrations in the samples were calculated with reference to the diameters of the clear zones around the standard NJ solutions.

DETERMINATION OF STARCH IN FERMENTATION SAMPLES.

The sample (2ml) was applied to Dowex-50W x 2 (H^+ form) (2ml) and then washed through with water (6ml). The pH of the eluate was adjusted to 5.5 and its volume to 10ml. *Aspergillus niger* glucoamylase was added (45 units) and incubated at 40°C for 1 hour. A sample (10µl) was then taken and GOD-PAP glucose assay kit solution (1000µl) added before a further incubation of 30 minutes at 37°C. The absorbance of the solution was then read at 510nm in a Pye-Unicam SP1800 UV spectrophotometer against a blank of sample treated similarly but without the addition of the glucoamylase. The absorbances

were converted into starch concentrations with the use of a standard curve.

DETERMINATION OF DRY WEIGHT OF CELLS.

Samples (5 or 10ml) of fermentations were taken and acidified with an equal volume of 0.2M HCl. This was shaken, centrifuged (10 minutes at 3000 rpm in a Beckman JA20 rotor) and the supernatant poured off. The pellet was resuspended in 0.05M Tris-HCl pH 7.0 (5ml) and filtered through pre-dried and weighed Whatman glass fibre filters. The filter was then dried in a drying oven for 48h before being re-weighed.

3.2.2. FERMENTATIONS - COMPLEX MEDIA.

PREPARATION OF SPORE SUSPENSIONS.

Spore suspensions were prepared according to the procedure of Hopwood *et al* (1985a). The final concentration of spores was adjusted to 10^8 cfu\ml and the suspensions stored at -20°C.

LARGE SCALE FERMENTATIONS.

These were carried out by batch fermentation using a 2L glass vessel. The conditions were controlled by a Lifescience Bioreactor controller attached to the fermentation vessel. The fermentations were carried out in 1-1.5l of medium which was autoclaved *in situ* and inoculated with 5x30ml shake flask cultures (48h) in the same medium. Aeration was at 1l\min with stirring at 750rpm and the temperature was kept at 28°C. The antifoam agent polypropylene glycol was added at 1% when the compex soyabean medium was used.

SMALL FLASK FERMENTATIONS.

Aliquots (30ml) of medium were autoclaved and inoculated with spore suspensions (100µl - 10^7 spores) of the strain concerned and incubated at 28°C, 190rpm in an orbital shaker. All assays were performed separately on triplicate flasks, mean results are shown, thus indicating only trends.

TIME COURSE EXPERIMENTS.

For time-course experiments, flasks were removed at intervals during the fermentation and analysed as described in 3.2.1. Samples for dry weight analysis were removed before centrifugation (3000rpm for 10 minutes in a Beckman JA20 rotor) to obtain the supernatant for the other assays. The

trehalase assay was used for DNJ determinations. Supernatants were stored at -20°C in order for them all to be assayed at the same time. The dry weight analysis was carried out immediately.

THE EFFECT OF SLOW ADDITION OF GLUCOSE ON THE CONCENTTRATION OF DNJ PRODUCED BY *S.LAVENDULAE* 31434 AND *S.SUBRUTILUS* 445.

Fermentations of 445 and 31434 (6x30ml each) in soyabean medium with 4g\l D-glucose replacing the starch were carried out at 28°C, 190rpm in an orbital shaker. In half fermentations for each strain the glucose was all added at the start of the incubation but in the other half, the same total amount of glucose was added in 8 equal aliquots at 12h intervals from the start of the fermentation. The fermentations were stopped after 7 days, centrifuged as previously described and the supernatant used for determination of DNJ by GC.

EFFECT OF THE AMOUNT OF GLUCOSE IN SOYABEAN MEDIUM ON THE PRODUCTION OF DNJ BY *S.SUBRUTILUS* 445.

Soyabean medium was made up containing 0, 2, 4, 6, 8, or 10g\l glucose (3 x 30ml fermentations for each glucose level). After inoculation with *S.subrutilus* 445 spore suspension (100µl), the fermentations were incubated at 28°C, 190rpm in an orbital shaker for 7 days. After centrifugation at 3000rpm for 10 minutes (Beckman JA20 rotor), DNJ concentration in the supernatants was determined by the trehalase assay.

3.2.3. FERMENTATIONS IN DEFINED MEDIA. THE EFFECT OF NUTRIENT CONDITIONS ON THE PRODUCTION OF DNJ BY *S.LAVENDULAE* IN DEFINED MEDIA.

PRODUCTION OF DNJ IN DEFINED MEDIA WHICH ARE ALREADY DESCRIBED.

Aliquots (30ml) of the different defined media were inoculated with spore suspension of *S.lavendulae* 31434, *S.subrutilus* 445 or *S.lividans* TK24. They were incubated at 28°C, 190 rpm before being harvested. Upon being harvested, the entire culture was centrifuged at 3000 rpm for 10 minutes in a pre-dried and weighed Universal bottle. The supernatant was decanted and the concentration in it assayed by the GC

method (Section 3.2.1.). The pellet was washed with 0.05M Tris-HCl pH 7.0 (2 x 10ml) and then dried for 48h in a drying oven before being re-weighed. The media used were MGA, ISP4, ISP5, ISP7, which are already described, and ST01, a medium composed of various elements of defined and complex media used in this study. The contents of all the media are detailed in the Appendix.

TIME COURSE OF FERMENTATION OF *S.LAVENDULAE* 31434 IN MINIMAL GLUCOSE MEDIUM (MGA).

A large scale fermentation of 31434 in MGA was carried out as described in section 3.2.2.. 1L of MGA, autoclaved *in situ*, was inoculated using 4 x 30ml cultures of 31434 which had been inoculated from a spore suspension of 31434 and incubated at 28°C, 190rpm in an orbital shaker for 48h. Samples were taken at intervals and their dry weight analysed as in section 3.2.1.. The DNJ content was assayed by the trehalase method.

THE EFFECT OF CARBON SOURCE ON THE PRODUCTION OF DNJ BY *S.LAVENDULAE* 31434 IN DEFINED MEDIA.

Fermentations on a 30ml scale were carried out as follows. A basal medium containing 5.7mM K_2HPO_4, 2mM $MgSO_4.7H_2O$, 85.5mM NaCl and 1ml per litre of a Trace Element Solution

comprising 2.2% $FeCl_2$, 0.5% $ZnCl_2$, 0.25% $MnCl_2.4H_2O$, 0.105% $CuCl_2.2H_2O$, 0.1% NaI, and 0.05% $CaCl_2.6H_2O$ was supplemented with 10mM L-arginine and one of several carbon sources at 20mM. The pH of the medium was adjusted to 7.4 and autoclaved before being inoculated with spore suspension of 31434 (100µl, 10^7 cfu). The cultures were incubated at 28°C, 190rpm for 7 days. Upon harvesting they were treated as in the opening part of this section to find the dry weight and DNJ content of the fermentations.

EFFECT OF DIFFERENT NITROGEN SOURCES ON PRODUCTION OF DNJ BY *S.LAVENDULAE* 31434 IN DEFINED MEDIA.

The basal medium described above was supplemented with 20mM D-glucose and one of the nitrogen sources at a concentration of 10mM. The fermentations (30ml scale) were inoculated and incubated as above and upon harvesting were treated in the same way in order to assess the dry weight and DNJ content of the fermentation. The fermentation with L-asparagine as nitrogen source was also carried out in a second basal medium as used by Vilches (Vilches *et al.*, 1990) in order to see how this affected DNJ production.

EFFECT OF VARIOUS ARGININE:GLUCOSE AND PROLINE:GLUCOSE RATIOS ON THE PRODUCTION OF DNJ.

The same basal medium was used supplemented with nitrogen source (proline or arginine) at 10 or 20mM and D-glucose at concentrations between 0 and 100mM. The same procedure was used for conducting the fermentations and assaying the dry weight and DNJ content.

EFFECT OF VARYING THE PROLINE CONTENT OF THE DEFINED MEDIUM ON THE PRODUCTION OF DNJ.

The above procedure was repeated but with the D-glucose content of the medium constant at 20mM and the L-proline concentration varied between 0 and 100mM.

EFFECT OF INORGANIC PHOSPHATE CONCENTRATION ON THE PRODUCTION OF DNJ.

With the D-glucose and L-proline concentrations of the media constant at 20mM each, the phosphate concentration , in the form of K_2HPO_4, was varied between 0 and 40mM in the above procedure.

EFFECT OF AMMONIUM IONS ON THE PRODUCTION OF DNJ.

The above procedure was repeated with the phosphate concentration constant at 5.7 mM and with the addition of ammonium ions as $(NH_4)_2SO_4$ at concentrations of between 0 and 40mM.

EFFECT OF INITIAL PH OF THE MEDIUM ON THE PRODUCTION OF DNJ.

The same basal medium as above was used supplemented with 20mM D-glucose and 40mM L-proline. The pH of the aliquots of medium was adjusted to values between 5.5 and 9.0 prior to autoclaving. The fermentations and analyses were carried out as described above.

RESULTS AND DISCUSSION

PRODUCTION OF DNJ IN COMPLEX MEDIA. FERMENTATIONS.

The use of the trehalase assay on the samples taken from the 2L fermenter when the soyabean based media were used was a problem due to the solution going milky upon addition of the glucose assay kit solution. There was no such problem when the fermentations were carried out in the same medium on a smaller scale in shake flasks or in the fermentor with MGA and so the cause was probably the polypropylene glycol (PPG) antifoaming agent used with the complex medium in the fermentor. For this reason, the time-course studies were carried out in 250ml conical flasks containing 30ml aliquots of medium instead so that the addition of PPG was not needed.

TIME-COURSE STUDIES.

The study on the fermentation of *S.lavendulae* MB-733 in soyabean medium reported by Ezure *et al.* (1985) showed that NJ and DNJ were produced at different times during the fermentation. NJ was at a peak at 48h whereas DNJ was at its lowest at this stage and only reached a peak at 96h, by which time the NJ concentration was falling. The NJ peak value was about 700µg\ml and that for DNJ was around 150µg\ml. MB-733

was a high producing strain and although the *S.lavendulae* 31434 fermentation reflects the pattern of NJ and DNJ production seen in MB-733, the amount of DNJ produced is only about 35% of the level in MB-733.

In the 31434 fermentation, shown in Figure 3.2, the NJ was first detected at 53h peaked at 100h and had fallen to undetectable levels again by 166h. DNJ was not detected until 75h, and rose to 44.5µg\ml by 212h. It is interesting to note that soluble starch concentration was still over 70% of its initial value when DNJ synthesis began in contrast to the MGA and the improved defined medium where glucose was the carbon source and declined to much lower levels prior to DNJ biosynthesis.

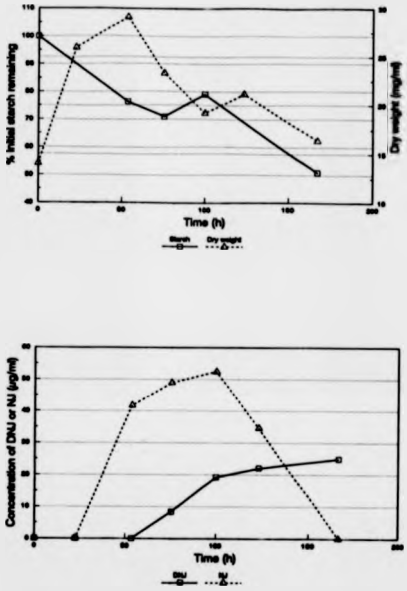


Figure 3.2. Fermentation of *S.lavendulae* 31434 in soyabean medium with starch as the major carbon source.

In contrast to the fermentation of 31434, that of *S.subrutilus* 445 showed no temporal separation of the production of DNJ and NJ. When starch was used as the carbon source (Figure 3.3), both were detected at around 100h when the amount of starch had been depleted to 50% of its original value. If the 20g\l soluble starch was replaced by 4g\l glucose (Figure 3.4), DNJ and NJ were detected at 92h and at this time, glucose had fallen to 14% of its original value. The fact that temporally the production occurs at the same time and that the production of DNJ is faster in the glucose containing medium indicate that glucose is probably not the only factor suppressing the production of DNJ earlier on in the fermentation. Levels of phosphate or nitrogen in the medium are probably also important. Although no additional phosphate was added, there may have been some in the soyabean medium which could contribute to suppression of DNJ production. The sodium nitrate added as a nitrogen source in addition to the soyabean is a more likely candidate as it will be utilised more quickly as a carbon source than the generally non-suppressive soyabean meal.

There are large differences seen in the NJ values from repeated fermentations of 445 in the soyabean medium with 4g\l glucose as the carbon source. The most likely reason for this is inaccuracy in the assay procedure. Due to the instability of the NJ, new aliquots of NJ bisulphite adduct were converted to NJ and standard solutions made up

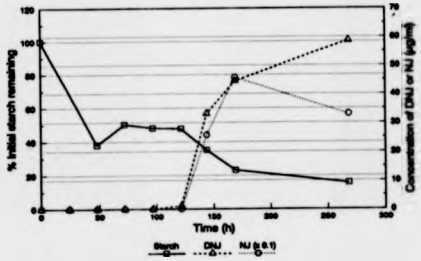
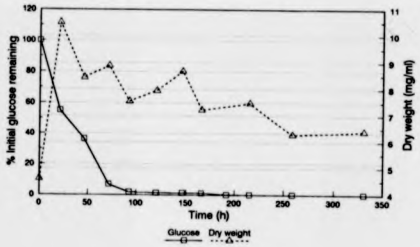


Figure 3.3. Fermentation of *S.subrutilus* 445 in soyabean medium with starch as the major carbon source.



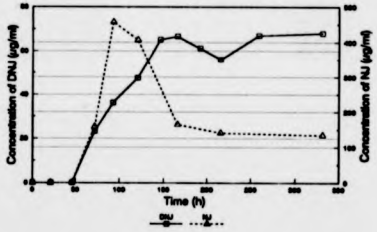


Figure 3.4. Fermentation of *S.subrutilus* 445 in Soyabean medium with glucose as the major carbon source.

from these each time an assay was performed. However, the lack of a single standard for use in each assay combined with the inaccuracy of converting and handing very small quantities of NJ would all have contributed to this problem. The values given therefore should be treated as indication of the trend of NJ concentration rather than its absolute value. The temporal differences in NJ production in 445 and 31434 could reflect differences in the regulation of the compound in the two strains. This could also explain the differences in the response of DNJ production in the two species to a slow glucose feeding regime, as shown in Table 3.1, if NJ is a precursor to DNJ. With slow glucose feeding compared to the addition of all the glucose at the start of the fermentation, the titre of DNJ in the 31434 fermentation after 7 days rose from 48.47 to 63.2 µg\ml and that in 445 fell from 46.26 to 27.8 µg\ml. The reason for this is again unclear but the fact that the amount of DNJ falls in 445 suggest that the level of glucose is kept at suppressive levels by the slow addition. In 31434 this might not be as important as NJ can still accumulate when at glucose concentrations repressing DNJ production and so when the production of DNJ is started, there is a large supply of NJ for quick conversion to the product.

Strain	Glucose addition	Concentration of DNJ (μg/ml)
31434	All at start	48.47 ± 8.4
	Stages	63.2 ± 12.1
445	All at start	46.26 ± 12
	Stages	27.8 ± 10.2

Table 3.1. Alterations in DNJ production due to slow-feeding of glucose to strains 445 and 31434.

EFFECT OF GLUCOSE CONTENT OF SOYABEAN MEDIUM ON THE PRODUCTION OF DNJ BY *S.SUBRUTILUS* 445.

The effect of adding different amounts of glucose to the soyabean medium without the addition of starch was investigated to assess the best medium for the isotopically labelled glucose studies. A medium containing only glucose as a carbon source was needed as if there were two or more carbon sources, the glucose, including the labelled glucose might be used for growth before the onset of DNJ production and so none would be available for incorporation into DNJ if glucose was to act as a precursor. The amount of labelled glucose available was also limited so that the medium giving the best yield of DNJ per unit of glucose added needed to be

found as well as the one giving a high enough titre of DNJ for enough to be produced for isolation and characterisation from a reasonable volume of medium. A target of 30mg DNJ in total unpurified medium was set so that around 10mg could be isolated as its acetyl derivative for NMR analysis.

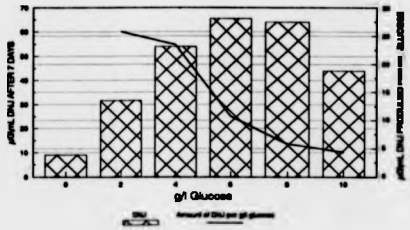


Figure 3.5. Effect of glucose concentration in Soyabean medium on the DNJ titre and "yield" from glucose.

The results, shown in Figure 3.5, demonstrate that the DNJ titre increased with glucose concentration up to 6 and 8g\l, where the DNJ titre reached 65.25 and 64.0 µg\ml respectively. With 10mg\ml glucose, the DNJ concentration only reached 44.0µg\ml after 7 days. This finding again

indicated that whereas high glucose concentrations may well repress the production of DNJ, sufficient is needed at the start of the fermentation to allow the establishment of a reasonable quantity of biomass to produce the DNJ.
The highest "yield" of DNJ (15.8 µg\mg glucose) was seen with only 2g\l glucose but to produce enough DNJ from this medium would need a litre fermentation. With 4g\l glucose, a small drop in the yield to 13.5 µg\mg glucose was seen but the titre of DNJ increased so that only a 600ml fermentation would be needed to get the required 30µg DNJ for isolation. As this volume would be easier to handle and the alteration in glucose concentration only resulted in a small fall in yield, the 4g\l glucose soyabean medium was used for the biosynthesis studies.

PRODUCTION OF DNJ IN DEFINED MEDIA.

DNJ PRODUCTION BY DIFFERENT STRAINS IN DESCRIBED DEFINED MEDIA.

The DNJ titre in the media tried was a lot lower than in the complex media used, as shown in Table 3.2. The highest DNJ titre was produced in medium ST01 (18µg\ml) which was derived from features of the other defined and complex media used. This figure is around 1/3 of the typical figures seen in the complex media.

MEDIUM	STRAIN	DNJ (µG\ML)
MGA	31434	10
	445	0
	TK24	0
ISP4	31434	13
	445	1.5
	TK24	0
ISP5	31434	0
	445	0
	TK24	0
ISP7	31434	0
	445	0
	TK24	0
ST01	31434	14.5
	445	0
	TK24	0

Table 3.2. DNJ titres in different defined media.

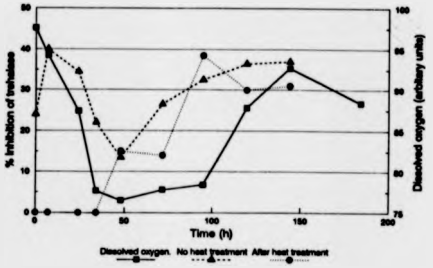
S.lavendulae 31434 was able to produce DNJ in 3 of the 5 media tested whereas *S.subrutilus* only produced it in 1 and even then at lower levels than 31434. *S.lividans* TK24 is a DNJ non-producer and so was used as a negative control in these experiments. The reason for the difference in the ability of these two normally producing strains to produce DNJ in these media is most likely to stem from them possessing slightly different mechanisms for the regulation of DNJ production. The difference has already been indicated by the temporal difference in the production of the hypothesised DNJ biosynthetic intermediate NJ in the two strains.

It was also observed that the only media where DNJ production by *S.lavendulae* 31434 was observed were the ones which used either glucose or the glucose polymer starch as the main carbon source. However due to the small number of media tested and the fact that other components were also varied makes it impossible to say how important this pattern may be. As medium ST01 gave the highest production of DNJ, this was used as a starting point for the studies on the effects of different medium components on the production of DNJ. *S.lavendulae* 31434 was also used as it was the organism giving the highest production in the largest selection of media.

PRODUCTION OF DNJ BY *S.LAVENDULAE* 31434 IN MINIMAL GLUCOSE MEDIUM (MGA).

The fermentation of 31434 in MGA (Figure 3.6) shows a typical two phase separation of cell growth (as measured by dry cell weight) and DNJ production. This kind of pattern is seen for the production of most secondary metabolites, especially in media supporting a fast initial growth rate. The production of secondary metabolites is usually restricted to times in the fermentation when the growth rate is less than maximal due to the depletion of 1 or more growth limiting substrates (eg. carbon or nitrogen sources or phosphate). The production of secondary metabolites during the growth phase can be encouraged by cultivating the organism in a medium which only

allows less than optimal growth, perhaps by the inclusion of a slowly utilised nitrogen source. In MGA, the production of DNJ by 31434 can be seen after 48h of incubation and has reached a maximum of 10.5 µg\ml by 95h. Comparison of the trehalase inhibition before and after heat\acid treatment indicates the presence of an acid\heat labile trehalase inhibitor on the fermentation from the time of inoculation to 48h. Although no bioassay was carried out on these samples, it seems likely, knowing that NJ is produced by this strain and is an acid\heat labile trehalase inhibitor, that this inhibition is due to NJ in the fermentation. The detection of NJ at t=0h in the



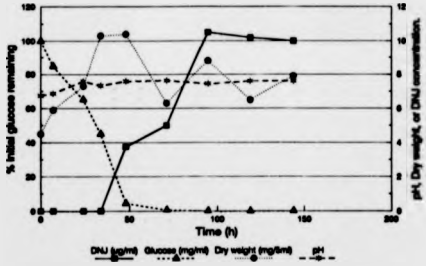


Figure 3.6. Fermentation of *S.lavendulae* 31434 in Minimal Glucose Medium (MGA).

fermentation can be explained by the presence of NJ in the small 48h fermentations used to inoculate the large fermentor. Another possibility is that the acid\heat labile inhibition of trehalase is due to proteases which degrade the trehalase being produced by the organism. However, since in control fermentations using the non-producing *S.lividans* TK24 no such inhibition was seen, this option seemed unlikely.

No DNJ was produced in the medium until the growth of the cells had virtually stopped and the level of D-glucose had become significantly depleted. It may be that earlier on in the fermentation, the high levels of glucose or growth rate of the organism prevent the production of DNJ. The production of NJ does not appear to be under the same kind of control, being produced earlier in the fermentation when both the growth rate and the glucose concentration are high. If NJ is a biosynthetic precursor to DNJ, one might expect it to be subject to the same type of regulation at a molecular genetic level. However, if the delay in the appearance if DNJ is due to the inhibition of a synthase involved in the conversion of NJ to DNJ, and not due to repression of synthesis of the expression of the biosynthetic genes, then the temporal difference in the production of the two inhibitors could be reconciled with this. The biggest rises in the DNJ titre (ie. the highest rate of production) were seen immediately after the onset of its biosynthesis, and before the glucose became undetectable in the medium. It should also be noted that the dissolved oxygen level in the fermentation was seen to fall

during the growth of the organism and was at its lowest during the production of DNJ. As the level of DNJ in the fermentation levelled off, ie. production came to a halt, the concentration of dissolved oxygen rose again. The changes in dissolved oxygen concentration during this period are, however, not significantly large to infer any relationship between them and the onset of DNJ production.

EFFECT OF CARBON SOURCE ON THE PRODUCTION OF DNJ IN DEFINED MEDIA.

The production of DNJ by *S.lavendulae* 31434 in the basic ST01 medium with various carbon sources was tested. A study on the effect of different carbon sources on the production of DNJ by *Bacillus subtilis* DSM 704 in a minimal salts medium with ammonium sulphate as nitrogen source has been described (Stein *et al.*, 1984) as has the effect of changing the carbon and nitrogen sources in a complex medium in its production by *Streptomyces lavendulae* MB-733 (Ezure *et al.*, 1985).

Carbon Source	Dry weight cells (mg/ml)	DNJ (µg/ml)	Specific DNJ production (µg/mg)
Glucose	0.58	5.84	9.5
Starch	0.63	3.13	4.96
Mannose	0.61	0	0
Glycerol	0.66	0	0
Sucrose	0.40	0.6	1.5
Lactose	0.29	0	0
Citric acid	0.22	0	0
No Carbon source	0.33	0	0

Table 3.3. DNJ production on different carbon sources in a defined medium.

The results of this study (Table 3.3) show that although glucose, soluble starch, mannose, glycerol and sucrose were all able to support the growth of *S. lavendulae* 31434, measurable amounts of DNJ were only produced when glucose, starch or sucrose were used. Although biomass was measured in the media with citric acid and lactose as carbon sources, the amount was no more than was seen in the control medium without any added carbon source in addition to the asparagine nitrogen source. The biomass produced in these media therefore probably reflects the utilisation of the asparagine as a carbon and nitrogen source. Although glycerol and mannose were found to yield the largest amount of biomass when used in the basal medium, neither was able to support

the production of DNJ. The significance of the limitation of the production of DNJ to media containing glucose, starch or sucrose may reflect a need for glucose units, or enzymes involved in, or a product of the metabolism of glucose in the biosynthesis of the DNJ molecule. Contrary to these findings, Ezure reported the production of significant amounts of DNJ in a complex medium containing either glucose starch or glycerol as the main carbon source (Ezure *et al.*, 1985). However as the medium used contained a complex nitrogen source, soyabean meal, the existence of other sugars such as glucose conjugated to components of the soyabean meal which could act as precursors in the biosynthesis of DNJ after the main carbon source is depleted cannot be discounted. Fructose, sorbitol and sucrose were found to support the production of DNJ by *Bacillus subtilis* DSM 704 at similar levels to glucose in a defined medium. When glycerol was used as carbon source, the amount of DNJ produced was only 2% of that produced on glucose (Stein *et al.*, 1984). Although fructose acted as a carbon source for the production of DNJ by *B.subtilis*, it did not yield significant quantities with *S.lavendulae* MB-733 (Ezure *et al.*, 1985), and was not included in the present study as *S.lavendulae* strains are unable to utilise fructose as a carbon source.

The observation that significant quantities of DNJ are generally produced on carbon sources which can provide either a glucose or a fructose unit may have implications for the biosynthetic pathway for DNJ.

It is interesting to note that in this study, slightly higher titres of DNJ were produced when glucose was the main carbon source instead of the glucose polymer starch. This might indicate that glucose is not exerting a significant amount of suppression on the production of DNJ. Starch, from which the glucose units would be released more slowly might be expected to avoid any glucose effects on the production of DNJ.

EFFECT OF NITROGEN SOURCE ON THE PRODUCTION OF DNJ IN DEFINED MEDIA.

Both inorganic and amino acid nitrogen sources were tried as sole nitrogen source in the defined medium. Of these, only L-asparagine, L-arginine, L-proline, urea and sodium nitrate were able to support the production of DNJ, as shown in Table 3.4. All of the nitrogen sources except L-valine were able to support significant growth of the organism. The highest specific production of DNJ (30.27µg\mg mycelium) was measured with L-proline as the nitrogen source and next highest (11.63µg\mg mycelium) was seen with L-arginine. The slower growth rate promoted by the proline carbon source provided a better environment for the production of DNJ than the arginine and asparagine, both of which supported a higher amount of cell growth.

Nitrogen Source	Dry Weight (mg/ml)	DNJ (µg/ml)	Specific DNJ production (µg/mg)
L-Asparagine	0.81	5.44	6.71
L-Tyrosine	0.47	0	0
L-Arginine	0.47	5.54	11.63
Sodium Nitrate	0.73	0.58	0.79
Glycine	0.48	0	0
L-Glutamine	0.32	0	0
Urea	0.57	3.58	9.74
L-Lysine	0.36	0	0
L-Proline	0.18	5.6	30.27
L-Valine	0.04	0	0
Ammonium Sulphate	0.14	0	0
DL-Aspartate	0.23	0	0
L-Leucine	0.44	0	0
L-Tryptophan	0.391	0	0
No Nitrogen	0.033	0	0

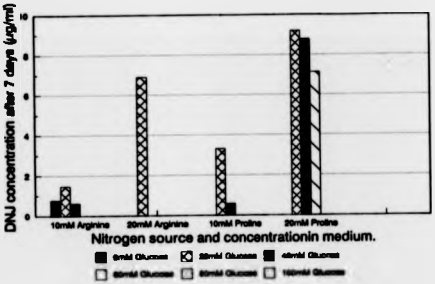
Table 3.4. DNJ titres in a defined medium supplied with different nitrogen sources.

The production of DNJ in the basal medium from ST01 was compared with that in one used by Vilches *et al.*(1990) to study the effects of different nutrients on the biosynthesis of oleandomycin by *S.antibioticus*. Added nitrogen and carbon sources were the same. Although the highest dry weight was seen in the Vilches medium, the most DNJ was produced in the ST01 base (2.95 and 6.71µg\mg mycelium respectively). The reason for this is unclear but it may be due to the higher

growth rate being incompatible with production of DNJ, or due to differences in the trace element solutions used in the two media.

EFFECT OF GLUCOSE:NITROGEN SOURCE RATIO ON THE PRODUCTION OF DNJ.

The glucose concentration was varied between 0 and 100mM in media containing 10 or 20mM L-arginine or L-proline. For both nitrogen sources at each concentration, the concentration of glucose giving the highest titre of DNJ after 7 days was found to be 20mM (see Figure 3.7). Furthermore this was also the concentration giving the highest amount of biomass. Proline again proved to be a better nitrogen source than arginine for DNJ production and the 20mM nitrogen source gave in both cases the most DNJ and the highest dry weight. The 20mM proline nitrogen source also allowed the production of DNJ in a medium containing a higher amount of glucose. 7.13µg\ml was produced on 60mM glucose in the medium containing 20mM proline whereas none was made with this glucose



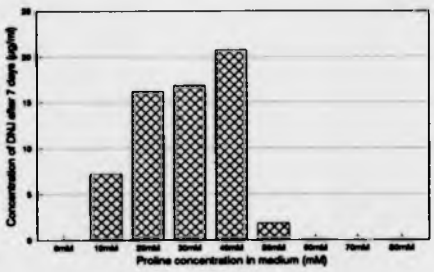
Nitrogen source	Glucose concentration (mM)	Dry weight (mg/ml)	DNJ (μg/ml)
Arginine (10mM)	0	0.4	0.76
	20	0.86	1.46
	40	0.65	0.6
	60	0.61	0
	80	0.45	0
	100	0.58	0
Arginine (20mM)	0	0.51	0
	20	0.68	6.9
	40	0.59	0
	60	0.52	0
	80	0.65	0
	100	0.44	0
Proline (10mM)	0	0.42	0
	20	0.92	3.3
	40	0.6	0.6
	60	0.50	0
	80	0.17	0
	100	0.16	0
Proline (20mM)	0	0.81	0
	20	1.12	9.19
	40	0.82	5.77
	60	0.74	7.13
	80	0.34	0
	100	0.26	0

Figure 3.7. The optmimum glucose:nitrogen source ratio for production of DNJ in a defined medium.

concentration on 10mM proline. A possible reason for this could be that in the medium containing the higher amount of proline, more growth and glucose consumption can take place before the growth is halted due to the nitrogen source running out. When this happens, there is less glucose left at the end of growth to suppress the production of DNJ.
Again, cell growth was seen when no glucose was added as a carbon source showing that proline was able to act as both carbon and nitrogen source under these conditions.

EFFECT OF PROLINE CONCENTRATION ON PRODUCTION OF DNJ.

As 20mM glucose was found to give the highest production of DNJ at both 10 and 20mM proline, this concentration was used for further studies. When the proline concentration was varied between 0 and 100mM, a different pattern was seen than that seen when the glucose concentration was varied. This is shown in Figure 3.8. The DNJ titre produced after 7 days incubation increased with proline concentration up to 40mM proline, with a maximum of 20.75µg\ml DNJ being produced at this concentration. The highest yield of biomass was with 20mM proline and no DNJ production at all was seen at proline concentrations of over 50mM.



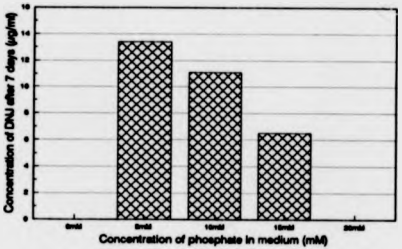
Proline concentration (mM)	Dry weight (mg/ml)	DNJ (μg/ml)	Specific production of DNJ (μg/mg)
0	0	0	0
10	0.84	7.2	8.6
20	1.28	16.16	12.6
30	0.82	16.8	32.3
40	0.43	20.75	48.5
50	0.37	1.8	4.8
60	0.34	0	0
70	0.40	0	0
80	0.49	0	0

Figure 3.8. Effect of varying proline concentration on production of DNJ in a defined medium.

EFFECT OF PHOSPHATE CONCENTRATION ON THE PRODUCTION OF DNJ.

As can be seen from the results in Figure 3.9, the concentration of inorganic phosphate in the medium has a marked effect on the production of DNJ by *S.lavendulae* 31434. No growth or production was seen if no phosphate was added to the medium which is not surprising since phosphate is needed for the synthesis of cell components such as nucleic acids and in intermediates in primary metabolic pathways such as glycolysis. The most DNJ was produced with the lowest of the other phosphate concentrations used, 5mM. At concentrations above this, less and less DNJ was produced and at 20mM phosphate, no DNJ was seen at all.

These results indicate that the phosphate suppresses the production of DNJ but it cannot be said whether this is likely to be due to inhibition of biosynthetic enzymes or due to their repression by the phosphate.



Phosphate concentration (mM)	Dry weight (mg/ml)	DNJ concentration (μg/ml)	Specific DNJ production (μg/mg)
0	0.88	0	0
5	1.85	13.4	13.79
10	1.88	11.1	6.26
15	1.24	5.6	4.6
20	0.80	0	0

Figure 3.9. Suppression of DNJ production in a defined medium by phosphate.

EFFECT OF ADDED AMMONIUM IONS ON THE PRODUCTION OF DNJ.

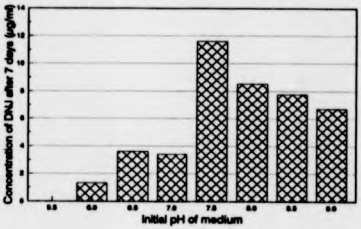
To see if DNJ production was suppressed by ammonium, different concentrations of ammonium ions, as ammonium sulphate, were added. The results are shown in Table 3.5. No DNJ was produced in media containing ammonium ions at 10mM or over and so it can be said that the production of DNJ suppressed by the addition of ammonium sulphate to the medium. It cannot be said whether it is due to inhibition or repression effects or whether it is due to a lowering of pH of the medium due to the import of ammonium into the cell. Ammonium sulphate was found not to be a good nitrogen source for the growth of this organism (see earlier) which could be for the same reason. Without any method of pH control, it is not possible to determine whether the effect seen here is due to the effect of the ammonium or a change in pH.

Ammonium concentration (mM)	Dry weight (mg/ml)	Concentration of DNJ (µg/ml)
0	1.38	16.2
10	0.44	0
20	0.43	0
30	0.51	0
40	0.58	0

Table 3.5. Inhibition of DNJ production by ammonium sulphate.

EFFECT OF INITIAL MEDIUM pH ON THE PRODUCTION OF DNJ.

Neutral and alkaline initial pH values were found to give the best production of DNJ and also the highest biomass, as shown in Figure 3.10. The optimum pH for DNJ production in this medium was 7.5 whereas the highest amount of biomass was seen with the initial pH at 9.0. The pH value of 7.5 may be most suitable because it provides a slower than optimum growth rate which is so often conducive to expression of the genes for secondary metabolite formation. A similar pattern is seen with the production of chlortetracyline. The optimum pH for the process is, at 5.8 to 6.0, slightly below the optimum pH for the growth of the producing organism (Weinberg, 1989). Although the data presented here give an indication of the best initial pH of the medium for a batch fermentation in which no pH control was being employed, a study involving fermentations under controlled pH conditions would be able to give details of the most beneficial pH for the medium during the actual phase of DNJ production.



Initial pH of medium	Dry weight (mg/ml)	DNJ concentration (μg/ml)	Specific DNJ production (μg/mg)
5.0	2.00	0.7	0.3
6.0	1.90	7.75	3.89
6.5	1.84	8.8	4.59
7.5	1.84	11.75	7.6
7.0	1.45	3.4	2.37
8.5	0.000	3.0	7.69
8.0	0.70	1.5	1.7
9.0	0.000	0	0

Figure 3.10. Effect of initial pH on the production of DNJ in a defined medium.

FERMENTATION OF *S.LAVENDULAE* 31434 IN THE IMPROVED DEFINED MEDIUM.

The time-course study in the improved defined medium (a combination of the best conditions from the defined medium studies) shows again that no DNJ is produced by the organism until a significant quantity of biomass has accumulated and most of the glucose has been depleted from the medium.

There was a longer lag phase before cell mass started rising compared to the fermentation in MGA, but this is probably due to the different inocula used. The MGA fermentation was carried out in a 2L Jar fermentor and inoculated with 48h grown cells, whereas this fermentation was carried out on small scale and was inoculated with spore suspension. There was a rapid increase in biomass, consumption of glucose and increase in DNJ titre between 90 and 135h and the curve of DNJ accumulation followed that of biomass accumulation fairly closely. The fact that DNJ synthesis was more closely coupled to cell growth in this medium may have been due to the change in the nitrogen source from asparagine, which would be assimilated fairly rapidly, to the more slowly assimilated proline.

The titre of DNJ at the end of the study (203h) was 14.8µg/ml and at 7 days incubation, it was 12µg/ml. Although this titre is less than was measured at 7 days incubation in the ST01 medium used in the initial defined medium study, it must be considered that the spore suspension for inoculating the

flasks, and the precise conditions of culture could have been slightly different in the two cases. The results of the different components of this study (eg. the effect of different nitrogen sources, or the effect of varying the proline concentration) are a better indication of the improvement made to the DNJ titre by these individual changes.

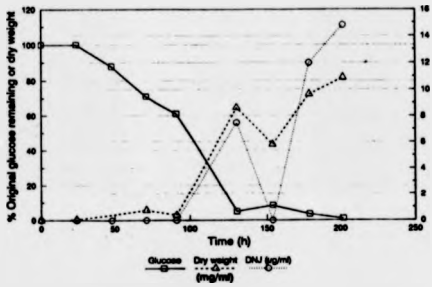


Figure 3.11. Fermentation of *S.lavendulae* 31434 in the improved defined medium.

GENERAL CONCLUSIONS.

Although the experimental data presented here represents only a very limited survey of the factors affecting the production of DNJ different media, it has resulted in the design of a defined medium which supports fairly diffuse growth of *S.lavendulae* and a level of DNJ production of about 25% of that in the soyabean medium. It was hoped to be able to use a defined medium for the isotope incorporation studies described in the next chapter, but as the production in such media was not sufficiently high to allow easy isolation of the product(s), a variation on the soyabean medium of Ezure *et al.* (1985), using glucose as the main carbon source was devised instead. The data in defined media also gives helpful pointers with respect to the biosynthetic pathway and how the synthesis of this metabolite may be regulated. It is clear that a complicated series of interacting factors control the growth rate of the organism and its production of DNJ. Inorganic phosphate is clearly a suppressive factor and ammonium ions might also be. Glucose was always very low in samples which were found to contain DNJ. In fermentations using glucose as the carbon source, the glucose was almost depleted before the onset of production, strongly implicating that this is suppressing DNJ production in the fermentations. On the other hand glucose is the carbon source which supports the highest DNJ titres out of the sources tested. This might be explained by the need for a supply of glucose units which

are readily accessible and not bound up in di- or oligosaccharides for the biosynthesis, or for enzymes or intermediates involved in the metabolism of glucose, in the biosynthesis of DNJ. Although glucose may be suppressive, it is also essential that glucose units are supplied to the growing cells as glucose, starch, or a disaccharide containing the glucose unit in order to create the right environment of enzymes or intermediates for the biosynthesis of DNJ.

It is interesting to note that the biosynthesis of streptomycin, an antibiotic which is derived from glucose, is also under glucose repression but is produced in a medium containing glucose as the main carbon source and soyabean meal as the nitrogen source (Shapiro, 1989).

Growth rate alone seems not to be an important factor in the control of DNJ biosynthesis since in some experiments, but not all, the conditions giving the highest DNJ titres were also the ones giving the highest amount of biomass.

CHAPTER 4. STUDIES ON THE BIOSYNTHETIC PATHWAY OF DNJ.

4.1. INTRODUCTION.

Although secondary metabolites are produced with a large and diverse range of structures from simple amino sugars such as DNJ to large macrolide antibiotics including the antihelminthic avermectins, they are synthesised from a relatively small number of precursors. Figure 4.1 shows how some of these precursors, derived from primary metabolism, are used to make different types of secondary metabolite.

In Chapter 1, DNJ and DMJ were introduced as plant-derived alkaloids, but they may also be considered as belonging to the aminosugars which are produced by microorganisms. The biosynthetic origins of these two families of compounds are, however quite different. Alkaloids are generally derived from amino acids such as tryptophan or lysine whereas the aminosugars and aminocyclitols of antibiotics are all synthesised from D-glucose. Either of these two origins could be applicable to the biosynthesis of DNJ and so is worth considering in more detail.

Figure 4.1. Major routes to secondary metabolites from primary metabolism.

Swainsonine is another glycosidase-inhibiting alkaloid which has been found in both plants and microorganisms. The biosynthesis of swainsonine in *Rhizoctonia leguminicola* has been shown start with lysine and is proposed to proceed as shown in Figure 4.2 (Harris *et al.*, 1988a).

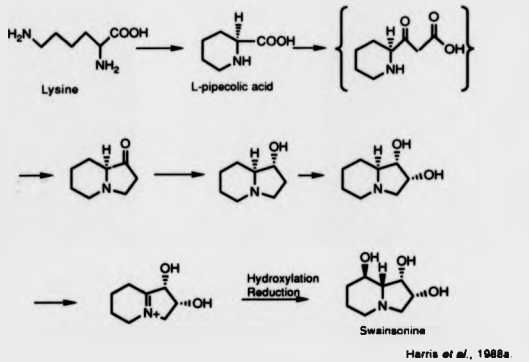


Figure 4.2. Biosynthesis of swainsonine from lysine.

Direct proof of the involvement of the bracketed intermediate is, at this stage, not available. The same pathway is also used for the biosynthesis of swainsonine in the locoweed *Astragalus oxyphysus*, although a branching of the pathway leading to the formation of another alkaloid, slaframine, is missing in the plant (Harris *et al.*, 1988b). Studies on the early stages of the biosynthetic pathway have shown that pipecolic acid is synthesised from lysine via saccharopine and Δ1,6-piperidine carboxylic acid (Wickwire *et al.*, 1990). An amino nitrogen from the precursor amino acid is incorporated into the ring system during the cyclisation. The pathway involves two stereospecific hydroxylations, at C-2 and C-8, and an inversion of stereochemistry at C-8a via the formation of the iminium ion.

Glucose is the biosynthetic precursor to the sugar components of the aminoglycoside antibiotics (Umezawa *et al.*, 1986). These antibiotics are made only of sugar units but other antibiotics, such as the macrolides also can have glycosidic components. The sugars used in antibiotics can be ones like mannose and glucose, taken directly from primary metabolism, or unusual sugars synthesised especially for, and used only in, antibiotics. Examples of the second type include the aminocyclitols streptidine and bluensidine, used in the construction of the antibiotics streptomycin and bluensomycin (see Figure 4.3) respectively.

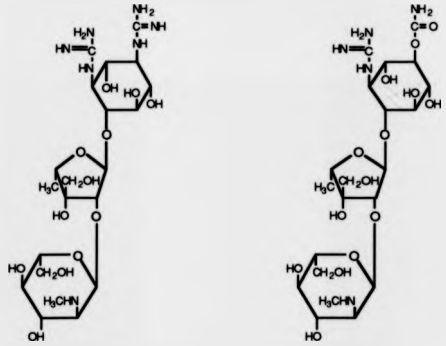


Figure 4.3.

During the biosynthesis of the aminocyclitols from D-glucose, the sugar backbone is incorporated intact into the product molecule, but its substituents can undergo a number of different transformations. These transformations include epimerisations, reductions, aminations, isomerisations, oxidations and dehydroxylations. They can be exemplified by the biosynthesis of streptidine shown in Figure 4.4.

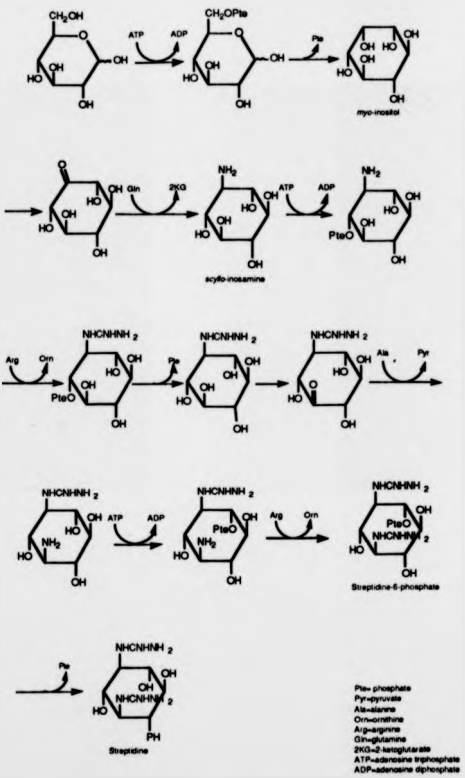


Figure 4.4. Biosynthesis of streptidine.

The initial conversion of the D-glucose to *myo*-inositol provides the cyclitol base which is used for the biosynthesis of other cyclitol moieties of aminoglycoside antibiotics. The *myo*-inositol is then oxidised by the enzyme *myo*-inositol:NAD+-2-oxidoreductase to provide a carbonyl group for the introduction of an amino group into the molecule. Amination of the precursor by glutamine is accompanied by an inversion of stereochemistry and yields *scyllo*-inosamine. *Scyllo*-inosamine is then phosphorylated at the 4-position, and amidino group transferred from arginine to the existing amino group and then the product is dephosphorylated again to give guanidinodeoxy-*scyllo*-inositol. The hydroxyl group at C-2 is then oxidised and alanine acts as a donor for the amino group then transferred to this position. Phosphorylation at the C-6 position and the addition of a second amidino group from arginine results in the production of streptidine-6-phosphate which is incorporated into the streptomycin molecule (Grisebach, 1978). The phosphate group is not removed from the streptidine unit until the final step in the streptomycin biosynthesis. This dephosphorylation activates the antibiotic and the introduction of the phosphate group is believed to act to protect the producing organism from an active form until it is ready for export out of the cell (Cundliffe, 1989).

When considering possible routes for the biosynthesis of DNJ, pathways from lysine (Fig. 4.5) as in the case of swainsonine, and D-glucose (Fig. 4.6) as in the case of the aminosugars, were hypothesised.

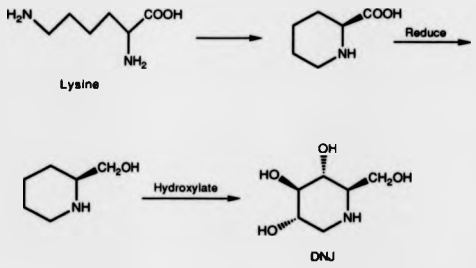


Figure 4.5. Hypothesised biosynthesis of DNJ from lysine.

In the route from lysine, the nitrogen is provided by the lysine and is incorporated into the ring during cyclisation, as in the biosynthesis of swainsonine, to pipecolic acid. The carboxyl group could then be reduced to a hydroxymethyl group and then three stereospecific hydroxylations at positions 2,3 and 4 would lead to the production of DNJ. A further hydroxylation at C-1 would produce NJ, but it is not hypothesised as a precursor in this route. Indeed, DNJ would

be more likely to act as a precursor to NJ if this pathway were in operation.

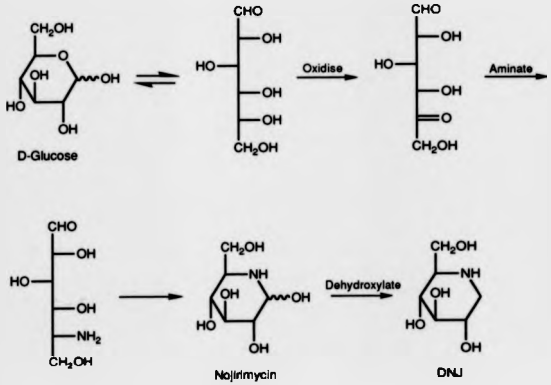


Figure 4.6. Hypothesised biosynthesis of DNJ from D-glucose.

The pathway from D-glucose does not require any stereospecific hydroxylations but just the introduction of an amino group at C-5 to yield NJ upon cyclisation, which can then be converted easily to DNJ. At the time of commencement

of these studies, nothing had been published on the biosynthetic pathway of DNJ apart from a mention that NJ was derived from glucose via an inversion of the sugar backbone (Yamaguch and Yonehara, 1967). However, it appears that full details of this finding were never published.

The finding that NJ was made in fermentations where DNJ was also produced (Ezure *et al.*, 1985) including that of *S.subrutilus* 445 (see Chapter 3), either before or at the same time as DNJ, and the similarity in these two compounds made the hypothesis that NJ was a precursor to DNJ reasonable. This, coupled with the mention of NJ biosynthesis from D-glucose and the aminosugar biosynthesis only from D-glucose, suggested that this sugar was the more likely precursor. Hence the isotope incorporation studies were started using isotopically labelled D-glucoses.

The biosynthetic significance of the presence of DMJ in DNJ-producing fermentations (Ezure *et al.*, 1988; Chapter 2, this thesis) was not established at this stage although the incidence of the DNJ, DMJ and NJ in the fermentations of both *S.lavendulae* 31434 and *S.subrutilus* 445 was taken as an indication that the same pathway was probably being used in the two strains, which are taxonomically very closely related.

Evidence from the nutritional studies in Chapter 3 also pointed towards D-glucose as the biosynthetic precursor for DNJ. Firstly, carbon sources supporting production of DNJ were ones which could provide a glucose unit to the organism,

with the more accessible forms of glucose allowing more DNJ production. Secondly, if lysine was a precursor for DNJ production, one might expect it to have some stimulatory effect on DNJ production when fed to the medium. No such effect was seen, in fact no DNJ production at all was seen when lysine was used as a nitrogen source.

All of the work described in this section was carried out in collaboration with David Hardick of the Department of Chemistry, University of Warwick, except the personal communications, which are his work alone.

4.2. MATERIALS AND METHODS.

4.2.1. ISOTOPE INCORPORATION STUDIES.

INCUBATION OF LABELLED PRECURSORS WITH *S.SUBTILUS* 445.

The medium used was soyabean medium with the soluble starch carbon source replaced by D-glucose at a concentration of 4g\1. DNJ concentrations at the end of the fermentations were assayed by the standard trehalase inhibition assay and by the gas chromatographic method, both of which are described in Chapter 2.

INCUBATION WITH [1-^{2}H]-D-GLUCOSE.

[1-^{2}H]-D-glucose (1g, 98 atm. %^{2}H, Aldrich) was used to make up 750ml of the medium. The remaining 2g D-glucose needed being made up with unlabeled material. The medium was divided up into 30ml aliquots and autoclaved. Each 30ml aliquots were inoculated with *S.subrutilus* 445 spore suspension (100µl, 10^{7} spores), incubated at 28°C, 190 r.p.m., for 7 days and pooled before isolation of the DNJ and DMJ. Analysis of the isotope incorporation pattern was then carried out.

INCUBATION WITH [1-^{13}C]-D-GLUCOSE.

[1-^{13}C]-D-Glucose (250mg, 98.7 atm. %^{13}C, obtained from Aldrich) was used to make up 125ml of medium. The remaining 250mg D-glucose was made up from unlabeled material. The fermentation was then carried out as above.

INCUBATION WITH [2-^{2}H]-D-GLUCOSE.

[2-^{2}H]-D-Glucose (0.5g, obtained from D. Hardick) was added to 1.5g of unlabeled D-glucose and used to make up 500ml of medium. The fermentation was continued as above.

FERMENTATION IN DEUTERIUM OXIDE.

The medium (100ml) was made up in deuterium oxide (100%) and divided into 30ml aliquots. The aliquots were autoclaved and then inoculated and incubated as for the [1-^{2}H]-D-glucose incubation. Unlabelled DNJ (20mg) was added to the fermentation prior to the isolation to facilitate the isolation of a sufficient quantity of DNJ for ^{2}H NMR analysis.

INCUBATION WITH [6,6-$^{2}H_{2}$]-NOJIRIMYCIN.

The medium was made up using 100% unlabeled D-glucose and divided into 30ml aliquots. After autoclaving, each aliquot was inoculated with *S.subrutilus* 445 spore suspension (100µl, 10^{7} spores) and incubated at 28°C, 190 r.p.m.. During the fermentation, [6,6-$^{2}H_{2}$]-NJ (obtained from D. Hardick) was added in two aliquots of 0.775 mg each 70h and 119h after inoculation. After 7 days, the fermentations were pooled and the DNJ\DMJ isolated and the isotope incorporation pattern analysed.

ISOLATION OF DNJ AND DMJ.

The isolation procedure used was as described in Chapter 2 up to and including the alumina column step. Sometimes the separation of the two compound achieved on the alumina was sufficient for analysis without further purification, or the DNJ\DMJ mixture was also sometimes subjected to analysis straight from this step. Alternatively, the two compounds were further separated on a silica column using 30% ethyl acetate in dichloromethane as solvent after having been acetylated.

ANALYSIS OF ISOTOPE INCORPORATION PATTERNS.

Positioning and incorporation of the isotopes was analysed using ^{13}C, ^{1}H and ^{2}H NMR which was carried out on a Bruker WH400, Bruker ACF 250MHz or a Perkin Elmer 220MHz spectrometer. Samples of acetylated DNJ or DMJ were run at 90°C in d-pyridine. Samples of free DNJ or DMJ were run in deuterium oxide at 25°C. Incorporations were also analysed using mass spectrometry. Mass spectra were run on a Kratos MS80 spectrometer using ammonia chemical ionisation.

4.3. RESULTS AND DISCUSSION.

Table 4.1 shows the results of the isotope incorporation studies.

Labeled substrate	% Substrate labeled	Labeled product(s)	% Product labeled	% Dilution of label
[1-^{2}H]-D-Glucose	33	[6-^{1}H]-DNJ $_{\beta}$ Pro-S proton [6-^{1}H]-DMJ $_{\beta}$ of C-6 labeled	21.7 21.9	66 66
[2-^{2}H]-D-Glucose	25	[6-^{1}H]-DNJ $_{\beta}$ Pro-R proton [6-^{2}H]-DMJ $_{\beta}$ of C-6 labeled	5.8 6.5	23 26
[1-^{13}C]-D-Glucose	50	[6-^{13}C]-DNJ [6-^{13}C]-DMJ	33	66
[6,6-^{2}H$_2$]-NJ	N/A	[6,6-^{2}H$_2$]-DNJ [6,6-^{2}H$_2$]-DMJ	N/A	N/A
^{2}H$_2$O	100	NONE	0	0

Table 4.1. Results of isotope incorporation studies.

The incorporation of ^{2}H and ^{13}C labels from D-glucose into specific positions in DNJ and DMJ during the fermentation of *S.subrutilus* 445 confirm that these compounds are both derived biosynthetically from D-glucose. The glucose in the medium at the start of the fermentation would have been used for the growth of the organism prior to being used in DNJ biosynthesis and so a reduced proportion of the original label in the medium would be recovered in the DNJ and DMJ. The results from the C-1 and the C-2 labeled glucose incubations also show that the biosynthetic pathway for DNJ and DMJ involves a head to tail (C-1 to C-6) inversion of the carbon backbone of the molecule, as reported for the biosynthesis of NJ (Yamaguchi and Yonehara, 1967). This evidence supports the hypothesis that NJ is a precursor to DNJ. The finding that both C-1 and C-2 labeled glucose incubations led to the incorporation of the label into the C-6 of DNJ and DMJ is consistent with an isomerisation of the glucose to fructose via an enediol during the biosynthesis of the two compounds. Such an isomerisation is shown in Figure 4.7. The transfer of the deuterium from the C-2 position to the C-1 position during the isomerisation results in its incorporation into the C-6 position of both DNJ and DMJ after the inversion of the sugar backbone. The biosynthesis of the aminosugar mycosamine, found in polyene macrolide antibiotics

has also been hypothesised to involve the formation of an enediol intermediate (Martin, 1977).

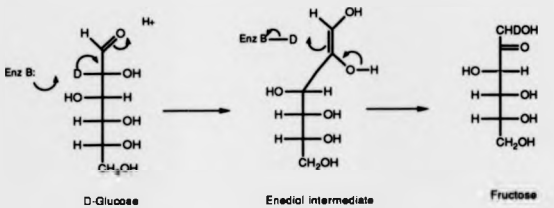


Figure 4.7. Glucose to fructose isomerisation showing movement of deuterium label from C-2 to C-1.

The involvement of glucose isomerase or glucose-6-phosphate isomerase, whose mechanisms both use a *cis*-enediol intermediate (Rose, 1975), is implicated by the pattern of deuterium incorporation seen with incubations with 1- and 2-deuteroglucoses. The deuterium from the C-1 of glucose was incorporated into the pro-S position at C-6 of DNJ and the one from the [2-^{2}H]-D-glucose was incorporated into the pro-R position of DNJ. This pattern is consistent with the one given by the catalysis of the isomerisation by either glucose isomerase or glucose-6-phosphate isomerase (Bock *et al.*, 1983).

The dilution of the label from [1-^{2}H]-D-glucose and [1-^{13}C]-D-glucose to 66% of their original value in the glucose added to the medium at the start of the fermentation probably arises from the use of glucose units from other sources eg. soyabean meal in the biosynthesis of DNJ. The finding that DNJ can be synthesised in the soyabean medium even when no D-glucose is specifically added to it (see Chapter 3, the effects of glucose concentration on DNJ production by *S.subrutilus* 445 in soyabean medium) supports this point. The glucose units required are probably present in the soyabean meal as glucosides which will only be liberated for use as a carbon source by the organism once the more accessible added source is well depleted. This source would only make up a very small proportion of the total available glucose at the start of the fermentation, but by the time that the biosynthesis of DNJ begins, the added glucose pool is depleted to such an extent that these other (unlabeled) sources of glucose are able to make up a much larger proportion of the glucose available for DNJ biosynthesis. It may be that in *S.lavendulae* 31434, where NJ is made early on in the fermentation, the dilution of the label could be much smaller due to its incorporation into the biosynthetic intermediates at a stage when the glucose pool contains a much larger percentage of label. Time did not permit investigation of this point.

When [2-^{2}H]-D-glucose is used, a much greater dilution of the deuterium label is observed, to approximately 23-26% of its

original value, compared to dilution to 66% with the C-1 labeled compounds. This could be due to exchange of the deuterium during transfer from C-2 to C-1 of glucose by the isomerase with an unlabeled proton from the solvent whilst bound to the enzyme. In an attempt to verify this, a fermentation was carried out with the medium made up in deuterium oxide (100%). If an exchange of the deuterium label with protons in the water was ocurring, as described, one would expect a fermentation in deuterium oxide to result in the incorporation of deuterium label into the DNJ produced. The lack of label in the DNJ isolated at the end of the fermentation was surprising and the reason for it unclear. The lack of incorporation may be due to a kinetic isotope effect, causing the incorporation of protons present in the medium due to exchange with groups on the media components in preference to deuterium from the deuterium oxide. It is worth noting that only a 2% incorporation of deuterium into fructose was seen when glucose was incubated with glucose isomerase in deuterium oxide at neutral pH, a percentage which increased slightly with increasing pH (Bock *et al.*, 1983), despite an earlier report that almost all aldose-ketose isomerases show some exchange with the solvent (Rose *et al.*,1975).

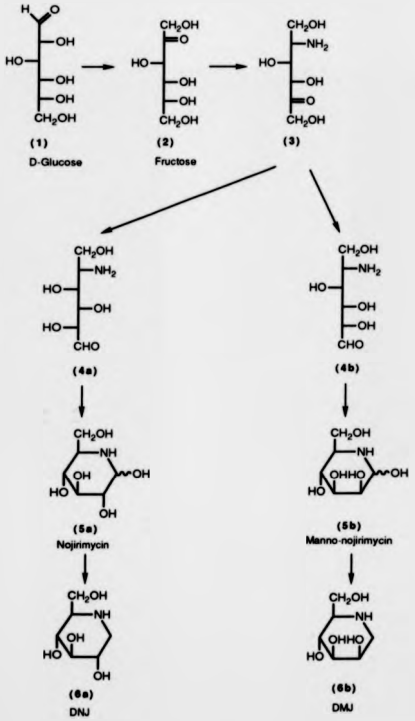


Figure 4.8. First pathway proposed for the biosynthesis of DNJ and DMJ from D-glucose.

On the basis of the above results, the pathway in Fig. 4.8 was proposed for the biosynthesis of DNJ and DMJ from D-glucose (Hardick *et al.*, 1991). Following the isomerisation of D-glucose to fructose, it was proposed that an oxidation at C-5 and an amination at C-2 would lead to the formation of (3). To avoid the incidence of a symmetrical intermediate which would presumably have resulted in the equal labelling at C-1 and C-6 of the DNJ, the amination would have to occur before the oxidation step. Alternatively, phosphorylation of the glucose at C-6 would lead to the required asymmetry, as would the the conjugation to a nucleoside diphosphate via C-1 as is seen for the biosynthesis of deoxysugars (Grisebach, 1978). If the transformation were to take place whilst the intermediate was still enzyme-bound, the problem would also be avoided.

Isomerisation of (3) to (4), in a reverse of the (1) to (2) conversion, could result in the formation of either (4a) or (4b), depending on which face of the carbonyl group in (3) was attacked. This would require the action of one enzyme which could act on either face of the carbonyl group, or two enantiospecific enzymes, each acting on only one of the faces. The linear forms would cyclise to give (5a) and (5b), with the inversion of the glucose carbon backbone ocurring due to the cyclisation occurring between the aldehydic group at what was C-6 of glucose and the amino group at the original C-2. This leads to the C-1 of glucose becoming the

exocyclic C-6 of NJ (5a) and NJ B (5b). These compounds are then dehydroxylated at C-1 to form DNJ (6a) and DMJ (6b). The occurrence of NJ and DMJ in the fermentations of *S.subrutilus* 445 can be explained by this pathway which also hypothesises the existence of *manno*-NJ (NJ B) in the fermentation, although this compound has not specifically sought during this study. It should be noted that NJ B has been co-isolated with NJ from *S.lavendulae* SF425 fermentations (Niwa *et al.*, 1984).

The role of NJ as a precursor to DNJ was confirmed by the results of the incubation with [6,6-$^2H_2$]-NJ. The label was incorporated into both DNJ and DMJ from the deuterated NJ indicating that either an epimerisation between the *gluco*- and *manno*- forms of NJ or DNJ was taking place, or that the transformation from (3) to NJ was operating in reverse so that (3) could then be converted to the DNJ branch of the pathway. The latter of the two could not be the case, since both of the C-6 protons of DNJ and DMJ were labeled from [6,6-$^2H_2$]-NJ, and this would not be the case if conversion back to the aldehyde group at the new C-6 had occurred.

Incubation with [5-^2H]-D-glucose led to the incorporation of the deuterium label into the C-2 position of DMJ without the labeling of DNJ (D. Hardick, personal communication) confirming that an isomerisation takes place from NJ B to NJ via a carbonyl group, resulting in the lack of label in the DNJ. Negligible incorporation of label into DMJ is seen when deuterium labeled DNJ is fed to the fermentation (D.Hardick,

personal communication) and so one can conclude that the epimerisation takes place at the NJ level. Furthermore, the observation that the label from the C-5 of glucose is incorporated into either of the products shows that there is no carbonyl group at the new C-2 in any intermediate prior to NJ B, thereby showing that (3) in figure 4.8 is not an intermediate.

In the light of the results from the incubations with [6,6-$^{2}H_{2}$]-NJ and [5-^{2}H]-D-glucose, the revised biosynthetic pathway shown in Figure 4.9 is proposed.

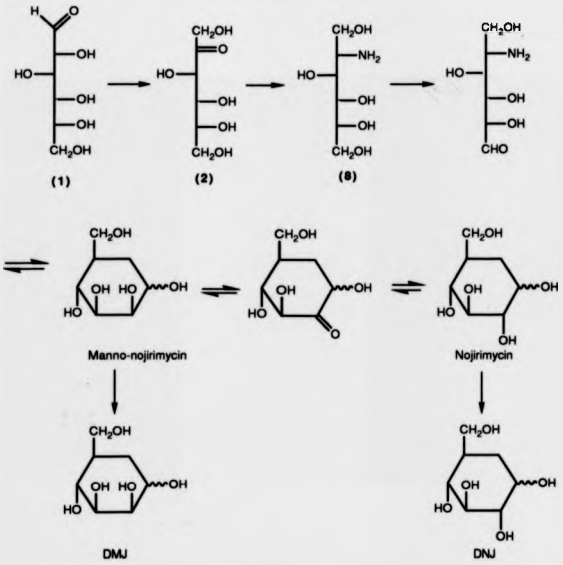
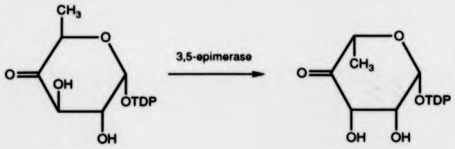


Figure 4.9. Revised biosynthetic pathway to DNJ and DMJ from D-glucose.

The order of the amination at C-2 and the oxidation at C-6 of fructose is unclear, as is the precise stage at which the

epimerisation takes place. It may be that the reaction takes place at the NJ B level only or at both the NJ B and the DMJ levels. The epimerisation cannot only be taking place at the DMJ level, or the NJ found in the fermentations of *S.subrutilus* would not be formed. The reaction could be catalysed by a single enzyme acting on both NJ B and DMJ, or on two different enzymes acting on one each. The proportion of the DNJ and DMJ produced therefore depends on the affinity of the epimerase for the *manno-* form relative to the *gluco-* form. Epimerases have been found to be involved in the biosynthesis of 3,5-deoxysugars of gram negative bacteria (Melo and Glaser, 1968; Gaugler and Gabriel, 1973), and 3,5-epimerases are also involved in the biosynthesis of some aminosugar and aminocyclitol components of antibiotics, such as the streptose moiety of streptomycin in *S.griseus* (Wahl *et al.*, 1975). The reaction catalysed by the 3,5-epimerase in dihydrostreptose biosynthesis is shown in Figure 4.10.



Wahl *et al.*, 1975.

Figure 4.10. Reaction catalysed by 3,5-epimerase during the biosynthesis of the dihydrostreptose unit of streptomycin.

The question of the source of the amino group introduced into the molecule has not been addressed in this study. The most likely candidate for the amino donor is glutamine, which acts as an amino donor in the biosynthesis of streptidine. Alanine also donates an amino group for the biosynthesis of streptidine, but it is a weaker donor than glutamine, as is glutamate (Umezawa *et al.*, 1986).

In biosynthetic pathways from D-glucose, the transformations are very often carried out on the the sugar after it is conjugated to a nucleoside diphosphate, such as uridine diphosphate (UDP), or another group. Sugar nucleoside diphosphates are the precursors in the biosynthesis of deoxyhexoses in bacteria (Grisebach, 1978). Mycarose, for example, is synthesised as its TDP-derivative from TDP-glucose in *S.griseus* (Pape and Brillinger, 1973). The

biosynthesis of the streptidine component of streptomycin involves the phosphorylation of the intermediates rather than their derivatisation with a nucleotide diphosphate (see Fig. 4.4). Whereas the nucleosides are attached to the C-1 position of the sugar, the phosphate groups are added to the C-6 position.

The intermediates in the biosynthetic pathway of DNJ may also be derivatised. In the majority of the fermentations, the labeled compound was the precursor and only the final product was analysed and so intermediates could have been derivatised but no evidence of that is seen. The fact that deuterium was incorporated into DNJ and DMJ from underivatised deutero-NJ shows that if another group has been introduced into the molecule, such as a nucleoside diphosphate or a phosphate group, then it has been removed by the NJ stage. This is understandable since the free NJ is found in the fermentation of *S.subrutilus* 445. If the intermediates in DNJ biosynthesis are derivatised it is unlikely that they have a nucleoside diphosphate group attached at the C-1 position as this would be expected to interfere with the action of the isomerase during the glucose to fructose isomerisation. Phosphorylation at C-6 is more likely but the intermediate would have to be dephosphorylated prior to the oxidation of that group to the aldehyde and the formation of the NJ B. The inclusion of a dephosphorylation step may lead to the same kind of inhibition of product formation by the inhibition of a phosphatase as seen in the biosynthesis of streptomycin. In

the nutritional studies, inorganic phosphate was found to inhibit the production of DNJ in *S.lavendulae* 31434, which probably follows the same pathway. Alternatively, the complete biosynthesis of DNJ could take place without and such derivatisation of the intermediates at all.

In summary, it is clear that the biosynthesis of DNJ and DMJ from D-glucose in *S.subrutilus* 445 follows a pathway which shows many similarities with the biosynthesis of aminosugars and aminocyclitols in other *Streptomyces* and related organisms. This is in contrast to the biosynthesis of other alkaloids, such as swainsonine, in plant and fungi, which are made from amino acids including lysine and tryptophan. It would be very interesting to see whether the same path is used in the mulberry to make DNJ and in *Lonchocarpus sp.* to make DMJ.

Swainsonine biosynthesis follows the same pathway in both the fungus *Rhizoctonia* and the plant *Astragalus* (Harris *et al.*, 1988b). It has been hypothesised that this could be due to a transfer of the genetic information needed to make the swainsonine from the microorganism to the plant. A similar situation may be the case for DNJ and DMJ biosynthesis in plants and microorganisms. The soil provides an environment for the intimate contact between soil microorganisms, such as streptomycetes, and the rhizosphere of plants, facilitating infection and the transfer of genetic material between the two. Leguminous plants, of which *Lonchocarpus* is an example, are well known for their participation in such interactions.

If the same biosynthetic pathway were found to be used for the synthesis of DNJ in plants, then this would be good evidence for the transfer of the biosynthetic genes from the microoganism to the plant. The occurrence of DNJ in the mulberry and DMJ in *Lonchocarpus* might be accounted for by two different transfer events where genes for different parts of the pathway failed to be transferred or that the capability of making one of the compounds in each plant was lost during the evolution of the plant.

CHAPTER 5. ATTEMPTED CLONING OF THE BIOSYNTHETIC GENES FOR DNJ.

5.1. INTRODUCTION.

The development of recombinant DNA methodology suitable for application to *Streptomyces* and their production of secondary metabolites has led to the accumulation of important data concerning the organisation and regulation of genes involved in these processes, about their products, and their evolution. As with other areas of research on secondary metabolism, most of the information available is concerned with antibiotics, and a relatively small number of these have been studied in any great detail. The advantage of dealing with compounds with antibiotic activity is their ease of detection with a suitable indicator organism. This is particularly useful when screening for possibly only a single producing colony amongst thousands.

ORGANISATION OF ANTIBIOTIC BIOSYNTHETIC GENES.

Genetic analysis has shown that the biosynthetic genes for antibiotics are generally clustered (Jones, 1989; Seno and Baltz, 1989). The biosynthetic cluster for methylenomycin in *S.coelicolor* is situated on the SCP1 plasmid (Kirby and

Hopwood, 1977) but is the only example of a plasmid borne biosynthetic cluster found so far, the chromosome being the more usual location. Seno and Baltz (1989) do, however, hypothesise that some of the clusters which are believed to be located on the chromosome of the producing streptomycete will in fact turn out to be located on large linear plasmids also carried by some strains.

The biosynthetic clusters contain all of the genes encoding the biosynthetic enzymes in addition to any resistance determinants. In some cases there is a sub-clustering of the biosynthetic genes, with the genes for enzymes acting early on in the biosynthetic pathway being positioned together and apart from the genes acting later on in the pathway. The "late" genes may also be clustered together. Such internal organisation is seen in the biosynthetic gene clusters of antibiotics including tylosin (Seno and Baltz, 1989), actinorhodin, (Malpartida and Hopwood, 1984), and tetracenomycin C (Motamedi and Hutchinson, 1987). Sub-clustering is not a general pattern though and does not apply to the organisation of other biosynthetic genes such as those for chloramphenicol (Vats *et al.*, 1987) or undecylprodigiosin (Seno and Baltz, 1989).

One or more resistance genes is usually also clustered with those for the biosynthetic enzymes, along with positive or negative regulatory elements. The clustering of the biosynthetic and resistance genes may be important for insuring that they are all expressed at the same time and

that sufficient resistance is conferred to cover the amount of antibiotic being made. This organisation would also minimise the chance losing of the resistance gene without also losing the biosynthetic capability. An organism may possess two resistance mechanisms to a given antibiotic, one to protect against the antibiotic when it is made by the organism itself and another to protect against the antibiotic when it is encountered by chance after being produced by another organism. As these two situations would involve different levels of the antibiotic and possibly occur at different stages of growth, then the two resistance genes need to be under different types of regulation. This type of situation is seen with kanamycin resistance in the producer *S.kanamyceticus* (Seno and Baltz, 1989).

The clustering of the biosynthetic and resistance genes for an antibiotic has made their cloning and study considerably less difficult than if they had been spread randomly around the genome

STRATEGIES EMPLOYED FOR THE CLONING OF GENES INVOLVED IN THE BIOSYNTHESIS OF ANTIBIOTICS.

The cloning of a gene, or genes, initially involves the construction of a gene library in a suitable vector. The methods used in the construction of the library are basically the same for all cases, but the strategy used for the

screening of the library for the genes involved in antibiotic production depends largely on how much information, of a biochemical and genetic nature, is available on the antibiotic and its production. The construction of a gene library can use a bacteriophage, such as ϕC31, or a plasmid as a vector. Plasmid vectors are most often used and many are available in various sizes and copy numbers. The choice of vector for a cloning program is important since some can accommodate larger pieces of DNA than others and the existence of a gene in more than one copy might be lethal in some cases making the use of a single copy plasmid essential for the successful cloning of such a gene. Plasmids have been engineered from naturally ocurring plasmids including SCP2 from *Streptomyces coelicolor* (Lydiate *et al.*, 1985; Bibb *et al.*, 1980) to include markers for easy selection of transformants containing plasmids with inserts. For example, the often used pIJ702 has been engineered to contain the thiostrepton resistance gene from *S. azureus* and the melanin gene from *S. antibioticus* (Jones, 1989).

Construction of a gene library involves the partial digestion of the genome of the producing organism using a restriction endonuclease to produces fractions of the genome of different sizes. The pieces are then taken and ligated into the cloning site of a plasmid which has been cut with the same, or a compatible, restriction endonuclease. The cloning site of pIJ702 is located in the melanin gene so that insertional inactivation of this gene occurs when a DNA fragment is

ligated into it. Upon transformation of a suitable host, those containing plasmid can be selected for using the resistance to thiostrepton that they will have acquired from the plasmid and those with a plasmid with an insert by visually locating those thiostrepton resistant colonies which cannot make melanin.

Screening for transformants containing antibiotic production genes is carried out using a number of different methods, alone or in conjunction with one another. If little or nothing is known about the biochemistry or genetics of the production of the antibiotic in question, then the transformation of a non-producing host and screening the transformants for production of the antibiotic is one of the options available. This method requires the cloning of large pieces of DNA (at least 10Kb) to give the best possibility of including all of the biosynthetic genes, and any resistance determinants and regulatory elements, needed for the biosynthesis of the antibiotic without self-destruction of the producer. Although this procedure may be used alone to clone biosynthetic pathways, it is more likely to be used with other methods to verify that all of the cluster is included on a fragment of DNA which has been detected using one of the methods described below.

Blocked mutants are very useful tools for the study of both the genetics and biochemistry of the biosynthesis of an antibiotic and also in the cloning of the genes involved. A blocked mutant is a mutant form of an antibiotic producing

organism which has an aberration in one of the genes needed for the biosynthesis of the antibiotic. Because of the change in the gene, its product is no longer functional and so the antibiotic is not made. The mutation may be in a gene encoding an enzyme involved in the biosynthetic pathway, or in one of the regulatory elements. Feeding studies, where an intermediate produced by one blocked mutant is able to be converted into the antibiotic product by another mutant which is mutated in a different gene in the pathway can be used to put the mutants in order of the biosynthetic steps which they are blocked in. The analysis of the intermediates which build up in the blocked mutants will also allow identification of intermediates in the biosynthetic pathway. If a blocked mutant is transformed with vector carrying an intact copy of the gene which has the mutation and the gene is expressed, the production of the antibiotic will be restored. This complementation of the mutation can easily be screened for amongst transformants. In addition to the gene to complement this particular block, the cloned fragment may also contain other adjacent biosynthetic genes, which can be tested for by the complementation of mutants blocked in other genes, and may indeed contain the whole biosynthetic cluster and be able to confer production of the antibiotic on a non-producing species.

Complementation of blocked mutants is widely used in cloning strategies and has successfully been applied to the cloning of the biosynthetic clusters for actinorhodin form

S.coelicolor (Malpartida and Hopwood, 1984), bialaphos from *S.hygroscopicus* (Murakami *et al.*,1986), undecylprodigiosin from *S.coelicolor* (Feitelson and Hopwood, 1983), tetracenomycin C (Motamedi and Hutchinson, 1987) and cephalosporin form *S.clavuligerus* (Piret *et al.*, 1990), amongst others. Apart from being used to clone clusters of genes, the complementation of blocked mutants is used to analyse the gene content of fragments of DNA cloned by the other methods. The advantage of using the blocked mutant complementation method is that only one of the genes needs to be cloned to achieve a positive result, this gene can then be used as a probe to detect that gene in clones containing larger fragments of the producers DNA which will hopefully also contain additional parts of the cluster. No previous genetic or biochemical knowledge of the biosynthesis is required for the application of this method but the generation of the blocked mutants required can be tedious and difficult in *Streptomyces* (Jones, 1989).

If a resistance gene has been or can be cloned then the observation that resistance genes are often clustered with the biosynthetic genes can be used to find these biosynthetic genes in the same way that one gene found by blocked mutant complementation can be used to find others clustered with it. The initial cloning of the resistance gene requires the generation of a gene library from the producer in a host which is sensitive to the antibiotic and the screening of the transformants for resistant colonies. This method requires

that the resistance and antibiotic biosynthetic genes are clustered together and will not be applicable to the biosynthetic clusters of non-antibiotic secondary metabolites. Neither biochemical or genetic information is needed for this approach, which has been applied, for example, to the cloning of a 35Kb fragment of DNA from *Saccharopolyspora erythraea* encoding resistance to and production of erythromycin, cloned into *Streptomyces lividans* (Stanzak *et al.*, 1986), and in the cloning of the bialaphos biosynthetic cluster from *S.viridochromogenes* (Hara *et al.*, 1991).

If more of the biochemistry of the biosynthetic pathway is known, then it may be possible to screen for clones containing the gene for a specific biosynthetic enzyme. This can be done in a number of ways. Detection of the enzyme product can be carried out if a suitable biochemical enzyme assay type detection system is available, as in the cloning of the p-aminobenzoic acid synthetase of candicidin biosynthesis in *S.griseus* (Gil and Hopwood, 1983), or if an antibody to the enzyme has been generated to which a label can be attached. Both of these systems require that the cloned genes are expressed in the host, as do the complementation and total cloning approaches already described. A third method for looking for a clone containing a gene for a particular product does not require the expression of that gene but does need an amino acid sequence for at least some of the enzyme to be available. An

oligonucleotide probe is constructed with a sequence which corresponds to the amino acid sequence of part of the enzyme and then is used to probe a library for the gene. A 44b probe, corresponding to the amino terminus of the macrocin-O-methyl transferase, an enzyme involved in the biosynthesis of tylosin and its use to probe a genomic library in a bacteriophage vector led to the identification of a 40Kb fragment of DNA coding for 9 biosynthetic genes and a resistance determinant from the producer *S.fradiae* (Cox *et al.*, 1986). This approach has also been used in the cloning of the deacetoxycephalosporin C synthetase gene from *S.clavuligerus* (Kovacevic *et al.*, 1989).

The cloned genes for two enzymes in the biosynthesis of actinorhodin have been used to probe gene libraries from other polyketide antibiotic producers, who use very similar enzymes in their biosynthsis, and have successfully located biosynthetic genes for milbemycin in *S.hygroscopicus* and granaticin in *S.violaceoruber* (Malpartida *et al.*, 1987). This technique may also be applicable to the cloning of biosynthetic clusters for other families of antibiotics using similar enzymes.

When a plasmid is used as a cloning vector, integration of the cloned genes and vector into the host cell genome does not usually occur. When the cloned DNA has homology to part of the host cells chromosome, for example when the donor is a mutant form of the host strain, however, homologous recombination resulting in the incorporation of the vector

and insert into the host cell DNA can occur. The method of mutational cloning relies on this event and was used in the cloning of the methylenomycin (Chater and Bruton, 1983) genes which are situated on the SCP1 plasmid of *S.coelicolor* (Kirby and Hopwood, 1977). A gene library was constructed in phage ϕC31 KC400 which was then used to transfect the methylenomycin producer. If the ϕC31 KC400 contained a piece of a methylenomycin gene, then homologous recombination with the corresponding gene in the host would lead to the insertion of the vector as into the gene, thereby inactivating it. Screening for a disruption of methylenomycin production would therefore show which transformants had the virus integrated into one of the methylenomycin genes.

The strategy chosen for the cloning of genes involved in antibiotic biosynthesis depends on how much is known of the system. The approaches described above which involve the hybridisation of cloned DNA to an oligonucleotide probe do not require the expression of the gene in the host whereas those based on the detection of a product do. The expression of cloned genes in a heterologous host appears not to be subject to many restraints (Jones, 1989), but the choice of host is still very important since restriction systems in a heterologous system might degrade DNA which is foreign. In all cases, one must be aware of the possibility of switching on previously silent genes in the host by the introduction of foreign DNA. A silent phenoxazinone synthetase gene was activated in *S.lividans* by the transformation with cloned DNA

from *S.antibioticus* (Jones and Hopwood, 1984). The cloned DNA did not encode the phenoxazinone synthetase gene. Hybridisation studies using a cloned phenoxazinone gene have shown its presence in a silent form in other *Streptomyces* species indicating that silent genes might be common in this genus (Jones, 1989).

WHY CLONE GENES FOR THE BIOSYNTHESIS OF ANTIBIOTICS?

The analysis of cloned antibiotic biosynthetic genes can lead to the accumulation of a great deal of information about the biosynthesis of the product and its regulation. Identification of the location of, and the effect of elimination of, the regulatory regions will indicate whether the pathway is under positive or negative regulatory control, or both. The sequencing of the biosynthetic genes will give information on the number of genes involved and comparison of the sequences with those of genes with a known function in a gene bank might also indicate the nature of the reactions that the gene products are catalysing.

On a more applied side, the identification of the regulatory elements and how they act might lead to the engineering of an overproducing strain for example by removing negative regulatory elements or increasing the number of elements having a positive effect on antibiotic production in the gene cluster. Finally, the cloning of antibiotic genes and the

application of the technology associated with it has led to the production of novel hybrid antibiotics (Hopwood *et al.*, 1985b).

5.2. MATERIALS AND METHODS.

5.2.1. ATTEMPTED CLONING OF THE BIOSYNTHETIC GENES FOR DNJ.

ISOLATION OF *S.LAVENDULAE* 31434 CHROMOSOMAL DNA.

S.lavendulae 31434 was inoculated as a spore suspension (100µl) into Tryptone Soya Broth (TSB, Oxoid) supplemented with 10% sucrose (30ml). After incubation at 28°C, 190 rpm for 2 days, the culture was centrifuged at 3000 rpm in a Beckman JA20 rotor for 15 minutes and the pelleted mycelium washed with 10.3% sucrose (2x20ml). The "total" DNA was isolated according to the following method, adapted from procedure 4 of Hopwood *et al* (1985a). Wet mycelium (50-100mg) was suspended in lysozyme solution (see Appendix, 500µl) and incubated at 37°C for 30 minutes. 2% SDS solution was then added (250µl) and the solution vortexed. The solution was then repeatedly extracted with neutral phenol-chloroform (see Appendix) until no white precipitate was seen at the interface. 3M Sodium acetate pH 4.5 (0.1 volume) and ethanol (2 volumes) were then added and the mixture left at -20°C overnight to precipitate the DNA. The precipitated DNA was pelleted by centrifugation in a microfuge at high speed for 10 minutes and then washed with 70% ethanol. After drying in a vacuum desiccator for 5 minutes, the pellet was dissolved

in T.E. Buffer (see Appendix, 500µl). All DNA preparations were inspected on a mini (25ml) agarose electrophoresis gel. Ones showing digestion of the chromosomal DNA were discarded. Chromosomal DNA samples were stored in T.E. buffer at 4°C.

QUANTIFICATION OF DNA.

The spectrophotometric assay of Hopwood *et al* (1985a) was used to quantify the DNA in the samples. Typically the solutions were found to contain 0.1 to 0.3µg\µl.

PARTIAL DIGESTION OF *S.LAVENDULAE* 31434 DNA.

Sau 3A restriction endonuclease (8µl, 8U) was added to a mixture of chromosomal DNA solution (100µl) and 10x E4 buffer (12µl) to start the digest. At 30 second intervals, aliquots (20µl) were removed from the incubation and added to a mixture of 0.5M EDTA pH 8.0 (1µl) and agarose gel loading dye (2µl). The samples were then run on a (0.8%) agarose gel (100ml) at 100V.

ISOLATION OF DNA FRAGMENTS FROM THE AGAROSE GEL.

A slice of agarose containing DNA fragments from the partial digest of 10Kb size and above was cut from the gel and the fragments eluted from this by the electrophoresis in dialysis tubing method of Hopwood *et al* (1985a). The fragments were stored at -20°C in T.E. buffer.

ISOLATION OF PLASMID pIJ943.

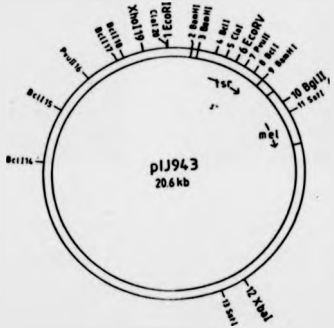


Figure 5.1. Plasmid pIJ943 (Hopwood *et al*., 1985a)

A strain of *Streptomyces lividans* TK24 containing the plasmid pIJ943 (see Figure 5.1) was grown in YEME (see Appendix) supplemented with 5% PEG 6000 and 5µg\ml thiostrepton for 72h at 28°C, 190 rpm. The cells were then centrifuged for

30 minutes at 10 000 rpm in a Beckman JA10 rotor at 20°C and the mycelium washed with 10.3% sucrose. The pellet was resuspended in 25mM EDTA, 25mM Tris, 0.3M sucrose (45ml) and lysozyme (50mg) added. After incubation at 37°C for 60 minutes, the appearance of the solution had become very mucosal and a freshly made up solution of 2% SDS in 0.3M sodium hydroxide (30ml) was added. Thorough but gentle mixing was followed by an incubation at 70°C for 15 minutes, until the solution had cleared. The solution was cooled and then the protein removed by repeated extractions with neutral phenol-chloroform. 3M Sodium acetate (7ml) and isopropanol (70ml) were then added, the mixture left at room temperature for 30 minutes and then centrifuged at 10 000 rpm for 20 minutes. The supernatant was decanted and the pellet resuspended in TE buffer with 10mM sodium chloride. 10mg\ml heat-treated ribonuclease in 0.3M sodium acetate pH 4.8 (50µl) was added and incubated at 37°C for a further 20 minutes. 3M Sodium acetate (1ml) was added prior extraction once with neutral phenol-chloroform (5ml) and twice with chloroform:isoamyl alcohol (24:1, 2 x 5ml). Isopropanol (10ml) was added to the aqueous solution and after 10 minutes at room temperature it was centrifuged at 10 000 rpm for 10 minutes. The pellet was dissolved in TE buffer (4.2ml) and 10mg\ml ethidium bromide (0.5ml) and caesium chloride (4.7g) added. The resulting solution was then centrifuged in a Vti 65 rotor at 20°C.

55 000 rpm for 18h and the band containing the plasmid DNA removed from the gradient after this time. This fraction was then extracted with isoamyl alcohol (4 x 0.5ml) to remove the ethidium bromide and then the aqueous layer made up to 0.5ml with distilled water. Ethanol (two volumes) was then added and after an incubation at -20°C overnight, the precipitated DNA was pelleted by centrifugation in a microfuge at high speed for 10 minutes. The pelleted DNA was washed with 70% ethanol (0.5ml), dried in a vacuum desiccator for 5 minutes and redissolved in TE buffer (20µl). Plasmid was stored in TE buffer at -20°C.

LINEARISATION OF pIJ943 AND REMOVAL OF 3' PHOSPHATE GROUPS.

Plasmid pIJ943 in TE (5µl, 15µg), 10 mg\ml ribonuclease (10µl) and Bgl II restriction endonuclease (50U) were incubated in 100µl final volume of a suitable buffer at 37°C for 1h. TE buffer (400µl) and Calf intestinal alkaline phosphatase (0.5U) added. After a further incubation of 1h at 37°C, the solution was extracted with neutral phenol-chloroform (500µl) and chloroform:isoamyl alcohol (24:1, 500µl). 3M Sodium acetate pH 4.8 (50µl) and ethanol (1ml) were then added and the solution left at -20°C overnight to precipitate the DNA. The precipitated DNA was pelleted by centrifugation at high speed in a microfuge for 10 minutes

and then washed with 70% ethanol before being redissolved in TE buffer (10µl). The linearised plasmid was stored at -20°C in the TE buffer.

LIGATION OF *S.LAVENDULAE* 31434 DNA FRAGMENTS WITH THE LINEARISED pIJ943.

Ligation reactions typically contained linearised and phosphatase treated plasmid pIJ943 (0.25µg), and chromosomal fragments from *S.lavendulae* 31434 (1µg) in a final volume of 50µl including T4 DNA ligase (5U). The ligation was carried out at 17°C overnight and the ligation mixture then stored at -20°C until used for transformation.

PREPARATION OF *STREPTOMYCES LIVIDANS* TK24 PROTOPLASTS.

S.lividans TK24 protoplasts were prepared according to the method of Hopwood *et al* (1985a).

TRANSFORMATION OF *S.LIVIDANS* TK24 PROTOPLASTS.

Freshly prepared protoplasts were used for each transformation since a large proportion were found not to survive the freezing and thawing process.

The protoplasts were transformed with the ligation mixture according to the method of Hopwood *et al* (1985a) and were plated onto dried R5 (see Appendix). After incubation for 24h at 30°C, the plates were overlayed with soft nutrient agar (SNA, see Appendix) supplemented with tyrosine and 100µg\ml thiostrepton.

Control transformations were also carried out using intact pIJ943 and also linearised pIJ943, both without any added chromosomal fragments.

SCREENING FOR PRODUCTION OF NOJIRIMYCIN BY TRANSFORMANTS.

Colonies growing through the thiostrepton containing overlay and not producing the pigment melanin were picked off the regeneration plates and transferred to RASS (see Appendix) plates containing 50µg\ml thiostrepton. After incubation at 30°C for 5 days, the spores were used to inoculate a 21cm x 21cm square plate containing nutrient agar (200ml). The plate was incubated at 30°C for 3 days and then overlayed with 70% nutrient agar seeded with *Micrococcus luteus* PCI 1001 (10^6

CFU/ml). After a further incubation at 30°C for 24h, the plate was inspected and colonies around which there was a zone of no *M.luteus* growth were investigated further.

BIOAUTOGRAPHY, TREHALASE INHIBITION AND GAS CHROMATOGRAPHIC INVESTIGATION.

Transformants giving a positive result in the above screen were grown in soyabean medium (30ml, see Appendix) for 4 days at 28°C, 109 rpm and then the cultures centrifuged to pellet the mycelium. The supernatants were subjected to the standard trehalase assay and gas chromatographic analysis, as described in Chapter 2, and to bioautographic analysis after TLC, as described in Chapter 3.

5.2.2. GENERATION OF BLOCKED MUTANTS.

MUTAGENESIS.

S.lavendulae 31434 spore suspension (7 x 100µl) was pelleted in a microfuge and then the supernatant removed and each pellet resuspended in sterile 0.2M phosphate buffer pH 7.0 (1ml). Ethyl methane sulphonic acid (EMS) was then added to

give concentrations of between 0 and 3% EMS. The tubes were sealed and whirlimixed before being incubated at 37°C for 1h. 0.16M sodium thiosulphate (9ml) was then added to neutralise the EMS. Serial 1 in 10 dilutions were prepared for each of the seven samples and then each dilution plated onto nutrient agar in triplicate (3 x 100µl). The plates were incubated at 30°C for 3 days and then the colonies which had grown counted. The EMS concentration giving only 1% survivors, compared to the control without any EMS, was used for the screening.

SCREENING FOR BLOCKED MUTANTS.

The spore suspension treated with 3% EMS was diluted to give single colonies upon regeneration at 30°C on nutrient agar plates. Colonies growing on these plates were picked off and put onto RASS plates and incubated for 4 days or until sporing well. Spores from these were transferred onto 21cm x 21cm square plates containing nutrient agar (200ml). After incubation overnight at 30°C, the plates were overlayed with 70% nutrient agar (150ml) seeded with *Micrococcus luteus* PCI1001 (10^6 cfu\ml). After incubation at 30°C for 24h, those mutants without a ring of *M.luteus* growth inhibition around them were screened again by the same method but with a 3 day incubation replacing the overnight one described above. Those still giving no inhibition of *M.luteus* were grown in soyabean

medium (30ml) for 4 days at 28°C, and then the culture supernatant subjected to bioassay and bioautography after TLC as described in Chapter 3.

FEEDING STUDIES WITH THE BLOCKED MUTANTS.

The blocked mutants detected in the above screens were streaked onto nutrient agar plates, three per plate in triangular arrangement, but not so that the growth would touch that of any of the others. These were incubated at 30°C for 3 days and then ovelayed with 70% nutrient agar seeded with *Micrococcus luteus* PCI1001 (10^6 cfu\ml). After a further incubation at 30°C for 24h, the plates were examined.

The feeding experiments were also carried out in liquid media. Pairs of the mutants were inoculated, in each combination, into soyabean medium (30ml) and incubated at 28°C, 190 rpm for 4 days. The mycelium were then removed by centrifugation and the supernatant used for bioassay and bioautography with *M.luteus* after TLC separation as described in Chapter 3.

5.3. RESULTS AND DISCUSSION.

ATTEMPTED CLONING OF THE BIOSYNTHETIC GENES FOR DNJ.

The lack of availability of information on the genetics or the biochemistry of, or mutants blocked in DNJ production meant that options for approaches to cloning the biosynthetic genes were limited. Although DNJ has no antibiotic activity, NJ, which has been shown to be a direct precursor of DNJ, has. It was initially planned to clone a resistance determinant to NJ and use that to probe a genomic library for the DNJ genes, assuming that they were clustered. However, the very limited availability of NJ which would be needed in significant amounts for screening for a resistant clone, coupled with the lack of sensitivity of non-producing *Streptomyces* strains tested with NJ made this approach unattractive. Even if enough NJ was available and a sensitive *E.coli* strain could be used as a host, the lack of sensitivity exhibited by *Streptomyces* could mean that they are not naturally sensitive and require no resistance determinant. Despite this, the bioactivity of NJ would provide a quicker method for screening for transformants harbouring genes for the DNJ pathway than would the trehalase assay for DNJ.

The only approach left was to try to clone the whole pathway into a heterologous non-producing host ad to screen transformants for NJ or DNJ production. *Streptomyces lividans*

TK24 was chosen as the host. It did not produce either DNJ or NJ and does not possess a restriction system which would be able to degrade any foreign DNA introduced into it. The plasmid pIJ943, a single copy number plasmid of 20.6Kb was also chosen for the protocol. The advantage of using a low copy number plasmid is that these are generally more stable when carrying large fragments of inserted DNA than are higher copy number ones.

Transformation frequencies seen for the ligation mixture into *S.lividans* TK24 protoplasts were typically in the order of 10 to 100 per µg of plasmid DNA, or around 10^{-4} of the regenerated protoplasts. This is a low frequency in terms of the plasmid DNA, 10^{7} per µg is quoted as a reasonable frequency for the transformation of *S.lividans* protoplasts (Jones, 1989). The proportion of regenerated protoplasts is not very low, indicating that the efficiency of the ligation and transformation procedures were generally not the main problem. If plasmid was probably being transformed into protoplasts which were then failing to regenerate, improving the regeneration frequency from around 10^{-5} would increase the number of transformants recovered. The cloning attempt was abandoned, for reasons which will be discussed later, before any attempt at improving the protoplast regeneration could take place.

Of the first 242 transformants isolated, inhibition of *M.luteus* PCI1001 was seen by three, transformants 2J, 13L and 13P. 2J was found to have an appearance very like that of

TK24, growing as a white colony which developed a red, non-diffusable pigment, but the other two were much more like the TK24 with the pIJ943 plasmid which grew as a white colony and then made a dark blue pigment which diffused into the medium. When *S.lividans* TK24 was initially tested in the screening assay, it gave no positive effect, but when the TK24 strain harbouring the plasmid was tried, an inhibitory ring was seen around the colony (see figure 5.2).

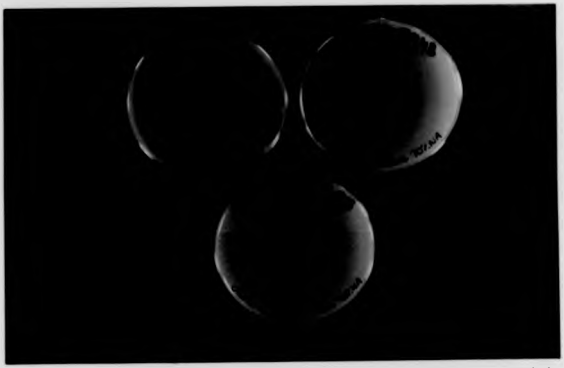


Figure 5.2. Inhibition of *M.luteus* by 2J and TK24 with pIJ943.

This result was highly unexpected since the plasmid contained no antibiotic determinants itself. There was the possibility that the presence of the plasmid in the cell was somehow causing the expression of a previously silent antibiotic

gene, an effect which is not unknown (Jones and Hopwood, 1984), or that the plasmid is causing a change in the environment in the cell so that a previously non-antibiotic metabolite was being converted into an active form. As the presence of the blue pigment, which was not melanin since it was produced on RASS which contains no tyrosine, correlated well with the inhibition of the *M.luteus*, this was the main suspect. The isolation of resistant strains of *M.luteus* from within the zone of growth inhibition around the *S.lividans* containing the pIJ943 was then attempted so that they could be used for screening without the interference of the inhibition effect due to the pIJ943. However, no *M.luteus* colonies grew within the zones, which is unusual if an antibiotic is involved, and so it was assumed that the inhibition by pIJ943 is not due to an antibiotic but probably to some other effect such as a change in pH preventing the growth of the indicator organism. A change in pH could also explain the change in pigmentaion between the red of *S.lividans* TK24 without the plasmid and the blue with it. Alternatively, it may have been that the effect was due to the tyrosinase (melanin) gene in the pIJ943. If the *M.luteus* was a tyrosine auxotroph, then the breakdown of all of the tyrosine around the colony would also have resulted in no *M.luteus* growth. The result in the previously mel$^-$ transformants could have been to a loss of the insert in the melanin gene resulting in the restoration of an active tyrosinase. It was assumed that the two transformants

producing the blue pigment were giving a positive result with *M.luteus* for the same reason as TK24 with the pIJ943 and so investigations on these strains were stopped but those on 2J were continued. The results are shown in Table 5.1.

	2J	TK24 + pIJ943	TK24
Trehalase assay	5%	0%	0%
M.luteus bioassay	-	+/-	-
Gas Chromatography	-	-	-

Table 5.1. Results of investigations on transformant 2J.

The results of the investigations on 2J show a pattern of results very similar to those seen for TK24 with pIJ943 and it is clear that the inhibition seen due to both of these strains is not due to nojirimycin. 2J shows no inhibition of *M.luteus* on the bioassay and although TK24+pIJ943 does, this is at an rf on TLC (as shown by bioautography - see Figure 5.3) which is very different to that seen for the NJ

5.3) which is very different to that seen for the NJ standard. No significant activity was seen against trehalase by 2J was seen either. The inhibition seen due to 2J could possibly have been a weak version of the inhibition exhibited by TK24+pIJ943 and indeed on prolonged culture, a slight blue coloration was seen around the substrate mycelium.

Figure 5.3. Bioautography of 2J fermentation supernatant.

It was also clear that this method was not suitable for screening for transformants in this system. It might be more applicable to a system involving a different plasmid, or for cloning back into blocked mutants, but its use here had to be abandoned. Time did not permit the setting up of another cloning strategy.

BLOCKED MUTANTS.

The same method as for the screening of the transformants was used to screen for mutants blocked in the biosynthesis of NJ. Spores of *S.lavendulae* 31434 were treated with the mutagen EMS at different concentrations and the one showing only 1% survival was screened for non-production of NJ. Spores were mutagenised instead of the mycelium,as this avoided the problem of having to fragment the mycelium into single cells before mutagenesis. As spores each contain only one copy of the genome, then any mutations cannot be masked by intact copies of the gene in other copies of the genome which might be present if incompletely fragmented mycelia were used.
After the primary screening of 2000 regenerated colonies with an overlay of *M.luteus*, after 24 h incubation, as shown in Figure 5.4, 39 putative blocked mutants were identified. When the incubation time was increased to 72h, prior to overlay, the 39 were reduced to only 8, the ones

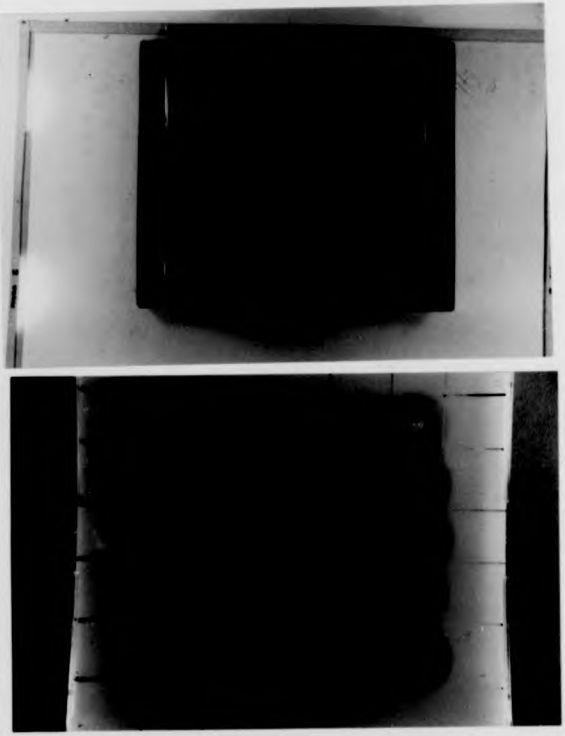


Figure 5.4. Examples of blocked mutants identified during the primary screen.

which were eliminated at this stage probably just being slower growing mutants which did not produce the NJ until later than 24h incubation. The 8 remaining mutants were then grown in soyabean medium and the supernatant from the cultures tested for bioactivity. Out of these, 4 were still not producing an antibiotic compound and so were used for cross-feeding experiments.

Mutant feeding experiments were first carried out on agar and the results showed little evidence of cross-feeding. A small halo of *M.luteus* growth inhibition was seen around the end of the growth of mutant 19I which was closest to mutant 10C on one of the plates, but this was not repeated on the other plate where these two mutants met. The inhibition was accompanied by a brown coloration of the area around the 19I growth where the inhibition was seen. None of the mutants isolates in this screen produced a pigment unless, as in this case, inhibition of *M.luteus* was also seen. Upon cross-feeding in the liquid media, all of the cultures containing 19I were coloured and all showed inhibition of *M.luteus* growth. Upon bioautography, it was clear that the inhibition in these cultures was due to NJ. In a culture containing only 19I, the same was seen showing that the inhibition in the others was due to a reversion of 19I to NJ production. No production of NJ was seen with any of the other crosses. These results are shown in figure 5.5.

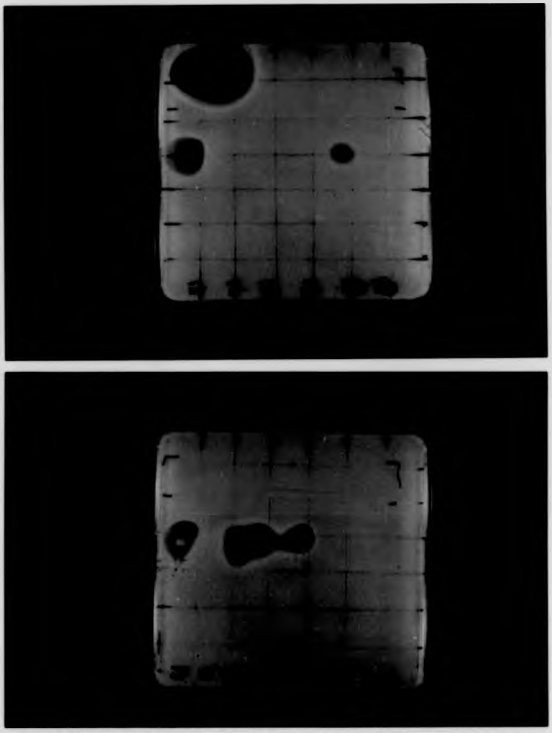


Figure 5.5. Results of blocked mutant feeding experiments in liquid media.

This lack of cross feeding may have been due to all of the mutants having their mutations in the same structural gene, or some regulatory element. A mutation in *a* regulatory element could result in no NJ production without actually producing an intermediate which could feed an earlier blocked mutant and result in NJ production. Alternatively, mutants not exporting intermediates would also not cross feed.

Whichever genes are mutated in these mutants, the likelihood is that they will be positioned with the biosynthetic genes and so complementation of the mutants would possibly provide a handle on the biosynthetic genes for cloning purposes. For any more than this however, a more comprehensive set of NJ blocked mutants would be needed and although the present work has not provided this, it does appear that the screen using detection of NJ by *M.luteus* is an easy and quick way to screen for such mutants.

CONCLUSIONS.

In this project, an enzymatic assay system for DNJ has been developed which avoids interference by NJ by chemically removing it from the sample to be assayed. NJ and DNJ inhibit a very similar range of enzymes and previous assay systems have only accounted for the presence of the other inhibitor in cultures by using an enzyme which is inhibited more strongly by one than the other (eg. Stein *et al.*, 1984; Ezure *et al.*, 1985). Pig kidney trehalase was used in this study. It was found to be inhibited by DNJ and NJ with Ki values of 3.43×10^{-6} and 2.6×10^{-5}M respectively. The Km value for the hydrolysis of trehalase by this enzyme was found to be 1.7mM, which compared well to the literature value of 2.1mM (Yonehara, 1987). The NJ was removed from samples by a heat and acid treatment and this allowed only the DNJ to be assayed. Results from this assay were comparable to those obtained from a gas chromatographic assay.

A known DNJ producer, *S.lavendulae* ATCC 31434 was obtained from the American Type Cultre Collection and the trehalase inhibition assay used to screen for other DNJ producers. *S.subrutilus* 445 was highlighted in the screen and found to produce both DNJ and DMJ. *S.lavendulae* also produces DMJ (Ezure et al., 1988).Comparison of the fermentation of 31434 and 445 revealed temporal differences in the production of NJ by the two strains. Whereas 31434 produced NJ early on in the fermentation, and DNJ later when growth had stopped and any

glucose in the medium had been much depleted, the production of both of these compounds by 445 was found to occur late in the fermentation.

Studies on the effects of culture conditions and nutrients on the production of DNJ in defined media showed that glucose, and starch were by far the best carbon sources for the biosynthesis and that proline was the best nitrogen source, of the ones tried. High levels of phosphate, ammonium sulphate and glucose were all found to supress the production of DNJ and the best initial pH for production in a batch culture was 7.5.

The biosynthetic pathway of DNJ and DMJ is closer to those of aminosugars in *Streptomyces* than the alkaloids produced by fungi. Glucose was shown to be the biosynthetic precursor to both DNJ and DMJ and a pathway hypothesised which proceeds via a glucose to fructose isomerisation. After amination and oxidation, epimerisation of NJ B produces NJ. The NJ and NJ B are dehydroxylated at C-1 to yield DNJ and DMJ respectively.

The importance of glucose and starch as carbon sources can be better understood in the light of these findings on the biosynthetic pathway as they provide the correct precursors and enzymatic environment for the biosynthesis of DNJ. Although glucose supresses DNJ production when it is present in high concentrations, it is still detectable when biosynthesis starts. DNJ titres stop rising when glucose becomes undetectable (see Figure 3.6, Fermentation of 31434 in MGA). The glucose consumed during the production phase

then probably goes partly to providing the metabolic energy for the cells but also to provide the precursors for the biosynthesis. The presence of glucose or starch early in the fermentation would ensure that all of the enzymes needed for the metabolism of glucose, including glucose isomerase which is hypothesised to be involved in the biosynthesis of DNJ (see Chapter 4), would be induced and present in the cell when DNJ biosynthesis starts. The finding that starch is not as good as glucose could reflect the fact that it provides a slightly less accessible supply of glucose units.

Attempts were also made at cloning the biosynthetic genes for DNJ, but interference with the screening assay caused by the presence of the cloning plasmid led to its abandonment. Mutants blocked in the biosynthesis of NJ were isolated though and will hopefully be used in genetic and biochemical investigations into the biosynthesis of DNJ which are being continued in the group.

APPENDIX

OATMEAL AGAR (per litre)

20g Fine Oatmeal

1g Yeast Extract

15g Agar

SOYABEAN MEDIUM

2% Soluble Starch

1% Soyabean Meal

0.05% KCl

0.05% $MgSO_4.7H_2O$

0.5% NaCl

0.2% $NaNO_3$

0.35% $CaCO_3$

pH 7.4

In some of the fermentations, the soluble starch in the above recipe was exchanged for D-glucose of various concentrations.

MINIMAL GLUCOSE MEDIUM (MGA) (per litre)

2g D-Glucose

1g L-Asparagine

0.5g K_2HPO_4

0.5g $MgSO_4.7H_2O$

1ml Trace Element Solution (T.E.S.)

pH 7.4

T.E.S.

1% $FeSO_4.7H_2O$

0.1% $CuSO_4.5H_2O$

0.1% $ZnSO_4.7H_2O$

ISP4 (per litre)

10g Soluble Starch

1g K_2HPO_4

1g $MgSO_4.7H_2O$

1g NaCl

2g $(NH_4)_2SO_4$

2g $CaCO_3$

1ml T.E.S.

Unadjusted pH 7.0-7.4

T.E.S.

0.1% $FeSO_4.7H_2O$

0.1% $MnCl_2.4H_2O$

0.1% $ZnSO_4.7H_2O$

ISP5 (per litre)

10g Glycerol

1g L-Asparagine

1g K_2HPO_4

1ml T.E.S.

pH 7.4

T.E.S.

0.1% $FeSO_4.7H_2O$

0.1% $MnCl_2.4H_2O$

0.1% $ZnSO_4.7H_2O$

ISP7 (per litre)

15g Glycerol

0.5g Tyrosine

1g L-Asparagine

0.5g K_2HPO_4

0.5g $MgSO_4.7H_2O$

0.5g NaCl

1ml T.E.S.

pH 7.3

T.E.S.

0.1% $FeSO_4.7H_2O$

0.1% $MnCl_2.4H_2O$

0.1% $ZnSO_4.7H_2O$

ST01 (per litre)

4g D-Glucose

1g L-Asparagine

1g K_2HPO_4

0.5g $MgSO_4.7H_2O$

5g NaCl

2g $NaNO_3$

1ml T.E.S.

pH 7.4

T.E.S.

2.2% $FeCl_2$

0.5% $ZnCl_2$

0.25% $MnCl_2.4H_2O$

0.105% $CuCl_2.2H_2O$

0.15 NaI

0.05% $CaCl_2.6H_2O$

LYSOZYME SOLUTION

2mg/ml Lysozyme

50µg/ml Ribonuclease (heat-treated)

0.3M Sucrose

25mM Tris (pH 8.0)

25mM EDTA

NEUTRAL PHENOL-CHLOROFORM

8-Hydroxyquinoline (0.05g) was added to phenol crystals (50g) and enough 0.1M tris buffer (pH 8.0) added to dissolve the solids. The upper, aqueous, layer was decanted and to the lower layer an aqual volume of chloroform:isoamyl alcohol (24:1) was added. This reagent was stored at 4°C in the dark.

SOFT NUTRIENT AGAR.

8g Difco nutrient broth

5g Bacto agar

1000ml Distilled water

100ml Tyrosine suspension (1.5g/L)

YEME.

3g Difco yeast extract

5g Difco bacto-peptone

3g Oxoid malt extract

10g D-Glucose

340g Sucrose

1000ml Distilled water.

After autocalving, add;

2ml $MgCl_2 . 6H_2O$ (2.5M)

25ml Glycine (20%)

TE BUFFER.

10mM Tris-HCl

1mM EDTA (pH 8.0)

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ERRATUM.

All slashes included in concentration units in the text are orientated in the wrong direction.

e.g. mg\ml should read mg/ml.